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(57) Abstract

The present invention provides a simple, cost-effective method, and kit, for enriching one or more target cells from a mixed cell population. According to the invention, target cells are detected by a detecting agent which attaches to an intracellular constituent of the target cell, for example, a nucleic acid, peptide, protein, etc., in the cytoplasm underlying the outer cell membrane or outer cell wall. The detected cells are then concentrated from the mixed population of cells using a solid phase support system which may include an immunoaffinity or immunomagnetic system. The enriched cells may then be identified and visualized using an identifying agent and a signal generating system. The present invention also provides a method for increasing the sensitivity of the enrichment of cells from a mixed population of cells by amplifying a selected intracellular constituent of the target cell prior to enrichment.

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SOLID PHASE ENRICHMENT OF INTACT CELLS USING INTRACELLULAR CONSTITUENTS

FIELD OF THE INVENTION

The present invention relates to the field of enrichment of a specific cell type present in a mixed cell population. The invention provides for concentration and identification of a target cell type, based on intracellular constituents, utilizing a solid phase support. The invention also provides a method for increasing the sensitivity of enrichment of target cells in a mixed cell population by amplifying a selected intracellular constituent present in the target cell.

BACKGROUND OF THE INVENTION

The technique of in situ hybridization is a powerful method for the detection and quantitation of nucleic acids and proteins at the level of a single cell. This includes a specific gene or gene product. The ability to detect the presence or absence of a specific gene product is important not only for genetic, biochemical, and molecular biological characterization of normal cell metabolism and differentiation, but also for the identification and detection of genetic markers for disease and infection. For example, in many cases, genetic diseases are characterized by the presence or absence of a specific gene product in the cell which is not present in normal cells. In addition, cells infected by

infectious agents also express nucleic acids and proteins that are not expressed in normal cells.

A problem with current methods for in situ hybridization is the inability to readily detect hybridized cells when they are in very low abundance in the cell population. Manual detection of cells requires scanning of microscope slides by skilled technicians which is laborious, time consuming and inefficient. Automated means for scanning large populations of cells for rare cells identified by in situ hybridization requires sophisticated and expensive computer-controlled optical or fluorescent scanning devices. In addition, specific cell types identified by in situ hybridization typically cannot be studied in isolation. Further, genetic, biochemical and molecular biological studies on the specific cells detected takes place in the presence of the entire population of cells.

Enrichment of rare cells is possible. Current methods for cell enrichment following in situ hybridization fall into two categories: immunomagnetic or immunoaffinity separation based on solid phase supports and flow cytometry employing fluorescence activated cell sorting.

In situ hybridization followed by concentration using solid phase supports is a common method for enrichment of cells based on detection of extracellular constituents such as the outer cell membrane or cell wall proteins or antigens. In this method, an extracellular protein or antigen is bound by an antibody that may be directly or indirectly coupled to a

solid phase support. Cells bound by antibody, coupled directly or indirectly to a solid phase support, are then enriched from the mixture of cells in suspension using immunomagnetic or immunoaffinity methods.

Although enrichment methods that employ solid phase supports are used to concentrate cells based on the presence of a specific protein or antigen on the cell surface, there are a number of situations where a protein or antigen on the outer cell surface or cell wall is not a suitable target for cell enrichment. In addition, cell surface antigens can be shed by cells and bind non-specifically to other cells, thereby reducing the efficiency of enrichment and increasing the proportion of non-target cells that are enriched. Current cell enrichment methods based on solid phase supports do not exploit unique intracellular genes and gene products.

Intracellular gene products can be used to characterize specific cells. During development and differentiation, normal cells typically express distinguishing nucleic acids and proteins in the cell cytoplasm. Many genetic diseases have been shown to be caused by, or can be diagnosed by, the appearance or disappearance of specific gene products located only in the cytoplasm or nucleus of the cell. Pathogenic and non-pathogenic viruses express specific viral nucleic acids and proteins found only in the cytoplasm. In addition, bacterial, insect and animal cells can harbor plasmids or viral vectors which contain recombinant DNA or expression products found only in the cytoplasm. Detection of these nucleic acids and proteins can be achieved by in situ hybridization. However, current methods do not provide a

means by which to enrich these cells following in situ hybridization.

It is possible, however, to enrich cells following detection by in situ hybridization of intracellular constituents using flow cytometry and flow sorting. See eg., M.L. Mendelsohn, The Attributes and Applications of Flow cytometry in Flow Cytometry IV Proceedings of the IVth International Symposium on Flow Cytometry (Pulse Cytophotometry) 15-27, (Laerum et al. eds. 1979) Universitetsforlateg, Oslo, Norway; L. Pajor et al., Biochemistry, 96:73-81 (1991); Y-L. Zheng et al., Prenatal Diagnosis, 15:897-905 (1995). In this method, intracellular nucleic acids or proteins are detected by in situ hybridization using probes that are directly labelled, or indirectly labelled, with a fluorochrome. The suspended cells are brought, one by one, to a detector by means of a flow channel where fluorescence is detected by a fluorescence cytometric sensor and the cells that fluoresce are sorted and subsequently analyzed. However, in addition to other disadvantages, flow cytometry followed by sorting requires skilled technicians and expensive equipment that is not portable.

Moreover, current methods for cell enrichment do not provide for increasing the sensitivity of detection when low numbers of cells or low levels of gene products are present.

The present invention provides a method for overcoming these and other shortcomings of current methods for cell enrichment.

SUMMARY OF THE INVENTION

The present invention provides a method for enrichment of a specific cell type in a mixed cell population. The invention also provides an enriched cell complex which is formed during enrichment of a specific cell type. Further, the components used in the invention may be combined into a kit for enrichment of a specific cell according to the method of the invention.

According to the invention, enrichment of a "specific" or "target" cell, which is present in a mixed cell population, is performed utilizing a solid phase support system.

The solid phase support system provides separation of an enriched cell complex from non-target cells present in the mixed cell population. As used herein, an "enriched cell complex" refers to the combination of a target cell, a detecting agent hybridized to an intracellular constituent of the target cell, and a solid phase support.

In general, the method of the invention may include fixing a mixed cell population with a fixing agent, permeabilizing the mixed cell population with a permeabilizing agent, detecting a "selected" or "target" intracellular

constituent of a target cell using a detecting agent and concentrating the target cells using a solid phase support.

Fixing agents suitable for the invention are known in the art. Preferred fixatives include those which act as cross-linking fixatives or precipitating fixatives. Cross-linking fixatives include, for example, formaldehyde, formalin, glutaraldehyde, formaldehyde-glutaraldehyde mixtures, α-hydroxyadipaldehyde, acrolein, dimethylsuberimidate and ethyldimethylamino-propylcarbodiimide. Precipitating fixatives include, for example, ethanol or methanol mixed with acetic acid or acetone and alcohol-ether mixtures.

Once fixed, the mixed cell population may then be permeabilized using a permeabilizing agent. According to the invention, a suitable permeabilizing agent is any compound which facilitates access of the below-described detecting agent to the cytoplasm of the cell and which does not inhibit enrichment of an intact and viable target cell. Preferred permeabilizing agents include those which unmask nucleic acids from associated proteins, form pores that allow access of the detecting agent to the cytoplasm or extract lipid from the outer cell membrane and allow access of the detecting agent to the underlying cytoplasm. Preferred permeabilizing agents for unmasking nucleic acids from proteins include, for example, Proteinase K, pronase E, dispase, diastase, papain, trypsin and pepsin/HCl for animal cells; cellulase or pectinase for plant cells; and lysozyme for bacterial cells. Permeabilizing agents that extract lipid from the outer cell membrane are known in the art and include, for example, alcohol such as

ethanol or methanol in combination with other compounds including acids such as acetic acid, or acetone. Other permeabilizing agents suitable for the invention include, for example, detergents such as sodium dodecyl sulphate, CHAPS™, Triton-X100™, Brij35™ and Brij58™. Moreover, some fixatives, such as formaldehyde and alcohol-based fixatives, also act as permeabilization agents and may make further permeabilization unnecessary. Permeabilization may also be accomplished using mechanical means such as freeze-thaw methods.

Another embodiment of the invention provides for increasing the sensitivity or detection of a target cell by amplification of an intracellular constituent when the cell, or selected intracellular constituent, is in low abundance. According to the invention, amplification of a desired intracellular constituent preferably is performed subsequent to permeabilization of the mixed cell population and prior to contacting permeabilized cells with a detecting agent.

After permeabilization, the mixed cell population is contacted with at least one detecting agent which has specificity for a target intracellular constituent which is present in a target cell of the mixed cell population. According to the invention, detecting agents may include genetic probes, antibodies, proteins, peptides, amino acids, sugars, polynucleotides, enzymes, co-enzymes, co-factors, antibiotics, steroids, hormones or vitamins. Generally, a detecting agent of the invention attaches to an intracellular constituent in a manner which is sufficiently stable for

concentration of the target cell using the below described solid phase support system.

Once the target cell is detected through the use of a detecting agent, the solid phase support system is used to concentrate the detected target cell. Target cells are concentrated by coupling the detecting agent to the solid phase support system. The detecting agent may be labeled such that the solid phase support system can detect and couple to the label of the detecting agent. Alternatively, a bound detecting agent may be detected and coupled to the solid phase support system without the need for labeling.

According to the invention, a solid phase support system includes a solid phase support and other components which may be necessary to separate the target cells from non-target cells in the cell population. Solid phase support suitable for a solid phase support system of the invention are known in the art and include, for example, magnetizable particles, silica, agarose, glass, dextran, fiber supports, cellulose and synthetic polymers, and similar supports. Preferred solid phase supports include superparamagnetic particles. A solid phase support system may also include a mechanism for separating the enriched cell complex from other cells in a mixed cell population, for example, in the case of a magnetizable particle solid phase support, a solid phase support system can include a magnetic field.

Once a target cell population has been enriched, target cells are eluted from the solid phase support. Because target

cell enrichment may lack complete specificity, eluted target cells may be further identified by using an identification system and visualized using a signal generating system. According to the invention, an "identification system" identifies the target cell using an identifying agent coupled directly or indirectly to a signal generating system. An identifying agent identifies a target cell by binding to a bound detecting agent or by directly binding to an intracellular or extracellular constituent of the target cell. Alternatively, an identification system may be the detecting agent which hybridized to an intracellular constituent prior to solid phase concentration.

A signal generating system provides visualization of an enriched target cell. The signal generating system may be incorporated into the identifying agent, or incorporated into a compound which binds to the identifying agent. If the identifying agent is the detecting agent, the signal generating system may or may not incorporated into the detecting agent prior to solid phase enrichment.

The invention also provides a kit for enrichment of at least one specific cell from a mixed cell population using a detecting agent specific for an intracellular constituent of a target cell. According to this embodiment of the invention, a kit may include a fixing agent, a permeabilizing agent, a detecting agent and a solid phase support system, as previously described. The kit may further include an identifying agent and signal generating system. The kit may include a single detecting agent for identification of a

single specific cell, or alternatively, multiple detecting agents for detecting multiple specific cell types or detecting a single specific cell type based on the presence of multiple intracellular constituents.

DETAILED DESCRIPTION OF THE INVENTION

The use of a solid phase support for concentration of a specific cell in a mixed population of cells provides a simple and cost effective means for enrichment of the specific cell from the mixed cell population. Preferably, the cells enriched are intact and viable for use in subsequent procedures.

The present invention provides an "enriched cell complex" and a method for enrichment of at least one specific cell, which may be present in a mixed population of cells, using a solid phase support system. According to the invention, a "specific" or "target" cell is enriched utilizing a solid phase support to concentrate cells having an intracellular constituent which is detected by a herein described detecting agent. Once a target cell is bound to the detecting agent, the cells may be separated from the mixed cell population using a solid phase support system. The combination of a target cell, a detecting agent and a solid phase support is referred to as an "enriched cell complex".

The invention not only yields an enriched population of target cells but also produces a mixed population of cells depleted of a specific cell type. The benefit of target cell

depletion in a mixed cell population is further discussed below. The invention also discloses a method for enhancing sensitivity of detection of a target cell by amplifying a "selected" or "target" intracellular constituent of the specific cell.

Enrichment of cells according to the invention, provides an enriched cell product which is useful in many areas of research and clinical use. As used herein, the term "enrich", and derivatives thereof, refer to a process for the treatment, detection and concentration of a target cell which may be present in a population of cells.

Typically, the population of cells on which the method of the invention is used will be a mixed population of cells. As used herein, a "mixed" cell population means a population of cells wherein one or more cells of the population has an identifiable characteristic which distinguishes the cell from one or more other cells in the population. Examples of mixed populations of cells include mixtures such as fetal cells and maternal blood cells; virally, bacterially, or fungally infected cells and non-infected cells; oncogenic cells and non-oncogenic cells; leukemic and non-leukemic cells; hematopoietic cells in bone marrow and blood; recombinantly transformed cells and non-transformed cells; differentiated and non-differentiated cells; cells expressing mutated genes and wild-type cells; and other cell populations which contain one or more cells with distinguishable intracellular characteristics. Mixed cell populations may be naturally occurring or artificially created for example, fetal cells

isolated from placental material and mixed into xenogenous blood or other fluids; human or animal cells from cell culture medium mixed into xenogenous blood or other fluids; or other artificial combinations of cells in liquid medium.

Typically, a mixed cell population of the invention is in a liquid suspension. Examples include cells present in blood, lymph, bone marrow, synovial fluid, amniotic fluid, cerebrospinal fluid, seminal fluid, ventricular fluid, nasopharyngeal mucous, sputum, semen, urine, water, effluent, sewage, feces, animal and plant cell culture media, bacterial and fungal enrichment media, tissue samples and tumors or organs disaggregated by physical, chemical or enzymatic means or other combination of cells in a liquid medium.

According to the invention, a "target" or "specific" cell include any cell which can be enriched according to the invention. Generally, a target cell will have one or more detectable intracellular characteristics which distinguishes the target cell from other cells present in a mixed population of cells.

Prior art solid phase enrichment systems typically rely on extracellular characteristics for identification of a target cell. However, while some cellular products, for example proteins, are distributed both intracellularly and on the cellular membrane, many proteins are only found intracellularly. Moreover, nucleic acids are generally not found as extracellular products. RNA, for example, is generally produced in the cell nucleus and transported to the

cytoplasm in eukaryotes. DNA is found primarily in the nucleus in eukaryotes. Hence, because many cellular products such as proteins or nucleic acids are exclusively found intracellular, or intracytoplasmic, in intact cells, they have not been considered attractive targets for enriching or depleting cells present in mixed cell populations using a solid phase support.

In contrast to prior art solid phase enrichment systems, the present invention does not rely on extracellular or outer membrane characteristics to enrich a target cell. Rather, the present invention provides for enrichment of a cell based on detection of one or more intracellular constituents. By "intracellular" it is meant that the target constituent is on the intracellular aspect of the outer cell membrane or cell wall. However, it is not required that the intracellular component be found exclusively intracellular.

According to the invention, an intracellular constituent includes, for example, nucleic acids, amino acids, proteins, peptides, polypeptides, lipids, metabolites, cofactors, polysaccharides, hormones or other intracellular component which may be detectable by a herein described detecting agent. In addition, the intracellular constituent need not be endogenous to the cell but rather, may come from an exogenous intracellular source, such as a plasmid, phage, virus, bacteria, protozoa, parasite, mycoplasma, fungus, or other similar source. Deoxyribonucleic acid (DNA), ribonucleic acid (RNA), peptides, polypeptides and proteins are the preferred

intracellular constituents for target cell enrichment according to the invention.

Problems that need to be overcome for enrichment of intact and viable cells based on intracellular constituents include, for example, maintenance of cell morphology, maintenance of nucleic acid integrity, maintenance of peptide or protein antigenicity, and accessibility of the solid phase support to the intracellular constituent. The present invention teaches that cell morphology, nucleic acid integrity and protein antigenicity can be maintained during fixation, permeabilization, in situ detection, and subsequent concentration using a solid phase support system. A further teaching is that following permeabilization of intact cells and in situ detection, there is sufficient access to the cytoplasm and sufficient detection agent in the exposed cytoplasm underlying the outer cell membrane or cell wall, to permit concentration of specific cells from a mixed population using a solid phase support.

Generally, cells enriched according to the method of the invention are morphologically intact. Preferably, the enriched cells are viable. As used herein a "viable" cell is a cell which is suitable for further study of anatomic, genetic, biological, morphological, physiological, pharmacological or other purpose for which the cell was enriched. Preferably, the specificity and sensitivity of the invention provides for enrichment of a low number of specific cells from large populations of mixed cells. However, where extremely low numbers of a target cell are present or the

intracellular constituent is present at low abundance in the cytoplasm, one embodiment of the invention provides for enhancement of detection of target cells by amplification of a selected intracellular constituent.

According to the invention, enrichment of a specific cell present in a mixed population of cells includes the steps of pretreating a mixed population of cells, fixing a mixed population of cells, permeabilizing the fixed cells, contacting the permeabilized cells with a detecting agent which binds to a selected intracellular constituent of a target cell and concentrating the target cell bound by the detecting agent using a solid phase support system.

I. Pretreating Cells

Cells of the invention may be pretreated in many ways. Preferably, the cells to be treated are in suspension. A cell suspension can be prepared using methods known in the art. For example, culturing cells in an appropriate medium including bacterial or animal cell culture media. Cell suspensions can also be prepared from body fluids such as blood, bone marrow, lymph fluid, and synovial fluid or can be prepared by disaggregating tissues, organs or tumors using known physical (eg. cutting, mincing, shearing, sieving or scratching adherent cells), chemical (eg. omission of divalent cations; with or without the addition of chelating agents) or enzymatic means (digestion with collagenase, dispase, trypsin, elastase, papain, pronase, hyaluronidase) to preferably

provide a single cell suspension. The cells prepared by these techniques may or may not be alive.

When an enzyme is to be used as a label, it may be necessary to inactivate the endogenous enzyme during pretreatment of cells. Peroxidases, for example, are inactivated with 1% hydrogen peroxide (v/v) in methanol for 30 min. Treatment of cells with 0.2M HCl for 30 min is sometimes used to improve the signal to noise ratio.

In general, the cell suspension is pelleted by centrifugation at 100 x g to 4000 x g, preferably about 400 x g, at 0°C to 25°C, preferably about 4°C, for 1-60 min, preferably about 15 minutes. The supernatant is removed and the cells are then fixed as described below.

II. Fixing Cells

According to the invention, a mixed population of cells is fixed with a fixing agent. As used herein a fixing agent is any compound which serves to provide a viable cell after enrichment (eg., prevention of osmotic damage, autolysis, etc.). Preferably, the fixative will result in the cell maintaining an accurate representation of the structure of the cell in vivo, and the cell will retain its original size with minimal loss of cellular materials during fixation. It is also preferred that the reactivity of intracellular constituents remains sufficiently high to enable them to be detected. The fixative chosen will depend on the material and probe being used and the level of sensitivity required. Any

fixing agent which does not prevent enrichment and which fixes a mixed cell population such that the enriched cells are intact and viable for the purpose for which the cells were enriched is suitable for the invention.

Preferred fixatives include those which act as cross-linking fixatives or precipitating fixatives. Preferred cross-linking fixing agents include, but are not limited to, formaldehyde, formalin, glutaraldehyde, formaldehyde-glutaraldehyde mixtures, α-hydroxyadipaldehyde, acrolein, dimethylsuberimidate and ethyldimethylamino-propylcarbodiimide. Preferred precipitating fixatives include, but are not limited to, ethanol or methanol mixed with acetic acid at a preferred ratio of 3:1, methanol mixed with acetone at a preferred ratio of 1:1, absolute methanol, 95% alcohol, and alcohol-ether mixtures.

In one embodiment of the invention, the cells may be fixed by resuspending cells in 0.4%-10% (w/v), preferably 4%(w/v) paraformaldehyde in phosphate buffered saline (PBS), (PBS=0.15M NaCl, 10mM Na-phosphate; pH 7.2), and incubating for 1 to 30 minutes, preferably 10 minutes at 0°C to 50C, preferably room temperature. Removal of the fixative can be accomplished by a series of 4-5 washes for 2-20 min each, preferably 5 min each wash, in PBS. Cells are pelleted by centrifugation between washes.

If the cells are not to be permeabilized immediately, the cells can be resuspended in an isotonic buffer such as PBS to a volume of 5-500μl, preferably 100μl at a concentration of

about 10^5 to 10^{12} cells per ml, preferably about 10^6 to 10^9 cells per ml. Once fixed, cells may be stored at 2°C to 12°C, preferably 4°C, for treatment at a later date.

III. Permeabilizing Cells

Cells are permeabilized using a permeabilizing agent. As used herein, a permeabilizing agent is any compound which facilitates access of a below-described detecting agent to the cytoplasm of the cell. Preferably, a permeabilizing agent does not inhibit enrichment of a target cell by the solid phase support. Any permeabilizing agent which provides enriched cells which are intact and viable for the purpose for which the cells were enriched is suitable for the invention. Preferred permeabilizing agents include those which unmask nucleic acids from associated proteins, form pores that allow access of the below described detecting agent to the cytoplasm, or that extract lipid from the outer cell membrane and allow access of the detecting agent to the underlying cytoplasm. Particularly preferred permeabilizing agents that unmask nucleic acid from protein include Proteinase K, pronase E, dispase, diastase, papain, trypsin and pepsin/HCl for animal cells; cellulase or pectinase for plant cells; and lysozyme for bacterial cells. Non-chemical means such as cycles of freezing followed by thawing of cells or microwave irradiation can also be used for permeabilization. Permeabilizing agents that form pores that allow access of the detecting agent to the cytoplasm include detergents such as sodium dodecyl sulphate, CHAPS™, Triton-X100™, Brij35™ and Brij58™. Permeabilizing agents that extract lipid from the

outer cell membrane are known in the art and include, for example, alcohols such as ethanol or methanol which may be used in combination with other compounds including acids such as acetic acid, or acetone. Some fixatives such as formaldehyde and alcohol-based fixatives also act as permeabilization agents and make further permeabilization unnecessary, but in general permeabilization is recommended.

In a preferred embodiment, fixed cells may be permeabilized with Proteinase K in PBS buffer at a concentration of 1-500µg/ml, preferably about 10-100 µg/ml for 1-180 min, preferably about 10 min, at 15°C-42°C, preferably about 37°C. As with other permeabilizing agents, the concentration of Proteinase K, the time of incubation and temperature used are optimized for each cell type. Permeabilization is stopped by replacing the Proteinase K solution with 0.02%-2% (w/v), preferably 0.2% (w/v) glycine in PBS for 1-20 min, preferably 2 min at 18°C-42°C, preferably room temperature. Stopping the reaction with glycine is an optional treatment.

Preferred permeabilizing agents for use with antibody detecting agents include alcohols such as ethanol and methanol which may be used in combination with compounds known in the art including acids such as acetic acid, or acetone (eg. methanol-acetone solution (1:1).

Once permeabilized, fixation of the cells may be repeated. Post-fixation is optional. The fixatives and methods previously described may be used for post-fixation of

cells. A preferred fixing agent is 0.4%-10% (w/v), preferably 4% (w/v) paraformaldehyde in PBS for about 1-180 minutes, preferably about 10 minutes at 0°C-50°C, preferably room temperature. Removal of the fixative can be accomplished by a series of 4-5 washes for 2-20 min each, preferably 5 min each wash, in PBS. Cells are pelleted by centrifugation between washes.

IV. Amplifying Cellular Constituents

Another embodiment of the invention provides for increasing the sensitivity of detection of a target cell by amplification of an intracellular constituent when the cell, or a selected intracellular constituent, is in low abundance. If increased sensitivity of detection is desired, preferably, amplification is performed at this stage of treatment.

In one embodiment, the invention provides for amplification of a selected nucleic acid which is present in low copy number in a specific cell. Preferably, the selected nucleic acid is amplified using the polymerase chain reaction (PCR). In situ PCR amplification on intact cells increases the amount of a selected nucleic acid and increases the sensitivity of detection of an intracellular nucleic acid.

Methods for carrying out PCR amplification of cells in suspension are known in the art. Such methods are described in, for example, J.J.O'Leary et al., J. Clin. Path. 47:933-938 (1994).

PCR Amplification takes place between two oligonucleotide sequences that are complementary to a defined segment of the selected nucleic acid sequence. See eg., G.R. Taylor, PCR: A Practical Approach, 1-13, (M.S. McPherson et al. eds. 1991) IRL Press, Oxford, England. For amplification of DNA products, cells that have been fixed and permeabilized, may be resuspended in 50µl-200µl, preferably about 100µl, of a buffer suitable for PCR amplification. Typically such a buffer will contain 10mM Tris-HCl (pH 8.4), 1mM-10mM $MgCl_2$, preferably 1.5mM $MgCl_2$, 5mM to 250mM KCl, preferably 50mM KCl, 50µM-200µM each dNTP, 1-2 units Taq polymerase, 100µg/ml gelatin and 0.1µM-0.5µM, preferably 0.25µM, each oligonucleotide primer (Tm >55°C, see below). The sample is overlayed with 75µl of mineral oil and the temperature raised to 90°C-95°C for 5-10 min preferably 5 min, to denature nucleic acids in the cells. The cells are then subjected to 25-35 cycles of 90°C-95°C for 15 secs to 1 min, preferably about 1 min; followed by 40°C to 60°C, preferably 55°C, for 30 secs to 5 min, preferably about 1 min; followed by 70°C to 75°C for 30 secs to 5 min, preferably about 1.5 min. Cycling is concluded with a final extension at 65°C to 80°C, preferably 72°C for 5-20 min, preferably about 5 min. The reaction is terminated by chilling to 4°C and/or by addition of EDTA to 10mM final concentration.

Typically, RNA targets are converted to DNA prior to PCR amplification by employing a reverse transcriptase enzyme such as Avian Myeloblast Virus (AMV) reverse transcriptase or Moloney Murine Leukemia virus (MoMuLV) reverse transcriptase.

Reverse transcriptase synthesizes a complementary DNA (cDNA) at the 3'-end of the poly(A)-mRNA strand with an oligo-p(dT)15 primer, or at non-specific points along the mRNA template with a random primer p(dN)6, or at a site determined by a specific primer. Reverse transcriptase synthesis is carried out using methods known in the art. Such methods are described in, for example, E.S. Kawasaki, PCR Protocols: A Guide to Methods and Applications, 21-27 (Innis MA., et al., eds. 1990), Academic Press, San Diego, CA. For amplification of RNA products, cells that have been fixed and permeabilized, are resuspended in a buffer suitable for reverse transcription. Typically, such a buffer may contain 50mM KCl, 20mM Tris-HCl (pH 8.4), 2.5mM $MgCl_2$, 0.1mg/ml bovine serum albumin, 1mM each dNTP, RNasin inhibitor (Promega Corporation, USA) at 1 unit/ml, 100pmol random hexamer oligonucleotides and 200 units of MoMuLV (or AMV) reverse transcriptase. The reaction is incubated at room temperature for 10 min and then at 37°C to 42°C for 30-60 min. The reaction is terminated by heating at 95C for 5-10 min. Following cDNA synthesis with reverse transcriptase, the cells are pelleted at 400 x g and a PCR reaction is carried out using specific primers to amplify the DNA sequence of interest as described above. To prevent diffusion of the newly synthesized nucleic acids from the cell following the PCR reaction, the cells are fixed as described above.

In addition, using methods known to those skilled in the art, labels can be incorporated into nucleic acids during the amplification process by substituting one of the nucleotides (dNTP) for a labelled nucleotide. See eg., A.R. Leitch et

al., In Situ Hybridization, Bios Scientific (1994), Oxford, England; B.D. Hames, et al., Nucleic Acid Hybridization: A Practical Approach, (1988) IRL Press, Oxford, England). Labelled nucleotides include, but are not limited to, for example, digoxigenin-nucleotides (eg. digoxigenin-11-dUTP), biotin-nucleotides, (eg. biotin-16-dUTP) and fluorochrome-nucleotides (eg. fluorescein-12-dUTP). According to this embodiment of the invention, if labels are incorporated during the amplification process, the below described detecting agent is the amplified nucleic acid sequence including a label. Target cells containing labelled nucleic acids can then be concentrated using a solid phase support system as described below. Alternatively, if the selected nucleic acid is not labelled during amplification, use of a below described detecting agent, such as a genetic probe, will typically be necessary.

An increase in detection sensitivity may be beneficial, for example, to detect and enrich cells infected with viruses such as HIV (human immunodeficiency virus) from mixed populations of cells. The presence of actively infected cells can be detected by amplifying viral RNA in the cytoplasm using reverse transcriptase and in situ PCR.

V. Detecting Agents

After permeabilization, preferably, the cells are sufficiently permeable to permit in situ detection of a selected intracellular constituent by a detecting agent. As

used herein, "in situ detection" refers to detection of an intracellular constituent in an intact cell.

According to the invention, an intracellular constituent is detected by using a detecting agent. A "detecting agent" is any compound presently known or later discovered which preferentially attaches to a "selected" or "target" intracellular constituent over other cellular constituents and which itself can be detected for coupling to a solid phase support. Typically, a detecting agent of the invention attaches to an intracellular constituent in a manner which is sufficiently stable for the below described solid phase support system to effectively concentrate the specific cells. The attachment may be reversible or irreversible, preferably reversible. Methods of attachment include, but are not limited to, ionic bonding, hydrogen bonding, covalent bonding, van der Waals interactions, and electrostatic interaction.

Suitable detecting agents, according to the invention, include genetic probes, antibodies, proteins, peptides, amino acids, sugars, polynucleotides, enzymes, coenzymes, cofactors, antibiotics, steroids, hormones or vitamins.

According to the invention, a detecting agent is preferably detectable by the below described solid phase support system.

A. Genetic Probe Detecting Agents

In one embodiment of the invention, a selected cellular constituent is detected by binding (hybridization) with a genetic probe as the detecting agent. A genetic probe is a substance that is used to identify a gene or a gene product and can be a genetic material such as DNA (deoxyribonucleic acid), RNA (ribonucleic acid) or synthetic oligonucleotides. Genetic probes may contain natural or chemical derivatives of the normal components of nucleic acids that include guanine, adenosine, uridine, thymidine, or cytosine. Utilizing a genetic probe, methods of in situ hybridization known in the art may be used to detect a selected intracellular nucleic acid. See eg., U.S. Patent No. 5,225,326 issued to Bresser et al.

If in situ PCR amplification is not required, the previously fixed cells are prehybridized (described below) to block any non-specific hybridization. Hybridization is then carried out by replacing the prehybridization solution with the same solution but now containing the hybridization probe.

When the detecting agent is a genetic probe, the probe may be rendered detectable by the use of a label prior to attaching to a selected nucleic acid or after attaching to the selected nucleic acid. Suitable labels to render a genetic probe detectable include, but are not limited to, digoxigenin, photodigoxigenin, biotin, photobiotin, 2-acetylaminoflourene, sulphone groups, mercury, fluorochromes, dinitrophenol, and

psoralen. Other labels include enzyme substrates, enzyme inhibitors and coenzymes.

1. **Types of genetic probes as detecting agents**

Genetic probes suitable for the invention can come from many sources. Genetic probes may be cloned nucleic acids. For example, a DNA fragment of interest may be inserted into a vector and amplified inside an appropriate host cell. The amplified DNA is then extracted and purified for use as a probe. Common vectors include bacterial plasmids, bacterial viruses, yeast artificial chromosomes and cosmids.

Genetic probes may also be synthetic oligonucleotides. A synthetic oligonucleotide of 15 to 50 base pairs, in length may be prepared using a DNA synthesizer. Genetic probes can also be prepared by amplification using the polymerase chain reaction, a process that relies on the use of suitable oligonucleotide primers that flank the DNA to be used as a probe. Probes can be double-stranded probes such as double-stranded DNA or complementary DNA (cDNA) or single-stranded probes such as single-stranded DNA or RNA or oligonucleotides.

2. **Labelling of genetic probes**

As stated above, a detecting agent is detectable by the below-described solid phase support system. Genetic probes may be made detectable by using labels. Labels can be incorporated into probes by enzymatic or chemical means. Double-stranded probes can be labelled by using DNA polymerases using known methods such as random primed DNA labelling, nick translation, labelling with Taq DNA polymerase

in the polymerase chain reaction. Single stranded probes M13, can be prepared by in vitro transcription for RNA probes using RNA polymerases such as T3, T7, SP6. Oligonucleotides can also be labelled by end-labelling or tailing.

Nucleic acid probes can be labelled enzymatically with a variety of labels. These include, but are not limited to, nucleotide derivatives of digoxigenin, biotin, and fluorochromes. In addition, nucleic acid probes can be labelled chemically with a variety of labels including but not limited to, photodigoxigenin, photobiotin, 2-acetylaminoflourene, sulphone groups, mercury, fluorochromes, dinitrophenol, and psoralen. Other types of labels include but are not limited to, enzyme substrates, enzyme inhibitors, and coenzymes chemiluminescers and bioluminescers. Methods to prepare labelled nucleic acid probes are known to those skilled in the art See eg., A.R. Leitch, et al., In Situ Hybridization, Bios Scientific, (1994) Oxford, England; B.D. Hames et al., Nucleic Acid Hybridization: A Practical Approach, IRL Press, (1988) Oxford, England).

In a preferred embodiment of the invention, the probe is labelled with a fluorochrome-nucleotide. The incorporation of a different fluorochrome-nucleotide into the each genetic probe allows simultaneous detection of more than one genetic probe in the same cell by use of an appropriate emission wavelength filter and epifluorescence microscopy.

The above methods involve the labelling of the genetic probe prior to hybridization with an intracellular

constituent of the target cell. However the genetic probe can be labelled following hybridization of the probe to the target using the technique of primed in situ labelling (PRINS) See eg., Koch J. et al., Genet. Anal. Techniques Applications 8:171 (1991). According to this method, DNA probes in the form of oligonucleotides, PCR products or DNA fragments are hybridized to a target cell nucleic acid. The hybridized DNA then acts as a primer for the incorporation of labelled nucleotides in situ. For labelling of DNA targets DNA polymerase is used. For RNA targets, reverse transcriptase is used to synthesize nucleic acid along the RNA template. Labels that can be incorporated into nucleic acid probes by enzymatic means have been described above.

3. **Prehybridization**

Prehybridization is an optional treatment used to minimize hybridization of the probe to non-specific target molecules. The prehybridization solution is generally identical to the hybridization solution described below but lacks the probe. According to the invention, cells are resuspended in a prehybridization solution containing, for example, 50% formamide, 2x SSC (1x SSC = 0.15M NaCl, 0.015M sodium citrate), 10% dextran sulphate and blocking DNA (or RNA) at 1 mg/ml. Blocking DNA (or RNA) is heterologous DNA (or RNA) that reduces non-specific hybridization by binding to molecules in the cytoplasm or nucleus that would otherwise bind probe or detection reagents. Other hybridization solutions suitable for the process of in situ hybridization are described below.

Prehybridization is preferably carried out at the same temperature as the hybridization reaction described below and is in the range of 30°C to 50°C, preferably about 37°C, for 30 min to 16 hours, preferably about 2 hours, to block non-specific binding of the probe. The temperature and duration of the prehybridization treatment are varied depending on the cell type and probe used. Following prehybridization, the prehybridization solution is removed after pelleting the cells.

4. **Probe and Target Denaturation**

Prior to hybridization, all double stranded nucleic acids must be denatured. Single-stranded nucleic acid probes and mRNA in the cytoplasm do not require denaturation. Denaturation is essential for double-stranded nucleic acid probes and for double-stranded DNA in the cytoplasm of the cell. Denaturation of the double-stranded nucleic acid probes and double-stranded DNA in the cytoplasm of the cell may be accomplished separately prior to hybridization. Methods for denaturing double stranded DNA include but are not restricted to, alkali or acid treatment, heat and organic solvents. See eg., A.R. Leitch et al., In Situ Hybridization, Bios Scientific, (1994) Oxford, England; B.D. Hames et al., Nucleic Acid Hybridization: A Practical Approach, (1988) IRL Press, Oxford, England.

The preferred method for denaturation according to the invention is combined denaturation of the double-stranded nucleic acid probes and chromosomal DNA in the presence of a hybridization buffer that contains a chaotropic agent such as

formamide (see below). Denaturation occurs at approximately 30°C above the melting temperature (T_m), usually at 70°C to 90°C, preferably 80°C, for 2-20 min, preferably 10 min, to denature the probes and chromosomal DNA in the nucleus. The T_m is defined as the temperature at which half the nucleic acids are present in single-stranded form. The T_m and reannealing of the nucleic acids is affected by temperature, pH, concentration of monovalent cations and the presence of organic solvents.

5. **Hybridization**

a. **Hybridization Solution**

In general, hybridization solutions may contain the following components.

(i) A chaotropic agent that decreases the T_m of nucleic acid hybrids and allows hybridizations to be performed at lower temperatures. This is desirable since cell morphology is adversely affected when cells are exposed to high temperatures over long periods of time. An example of a chaotropic agent which decreases the T_m of nucleic acid hybrids and allows hybridizations to be performed at lower temperatures is formamide. Hybridizations are generally performed at 30°C to 45°C with 30%-60% formamide present in the hybridization mixture. The presence of formamide also allows the denaturation of probes and cellular nucleic acids by heating to approximately 30°C above the T_m. Other chaotropic agents that can used include sodium iodide, urea, thiocyanate, guanidine, and perchlorate.

(ii) A monovalent cation, which stabilizes the hybrids once formed. Monovalent cations, such as sodium ion,

interact through electrostatic forces with the phosphate groups in nucleic acids. Electrostatic repulsion decreases with increasing salt concentration. High salt concentrations will stabilize mismatched hybrids and allow the detection of cross-hybridizing species.

(iii) A hybridization buffer. Typically, a hybridization buffer contains a 20-50mM phosphate, pH 6.5-7.5 buffer. Provided the hybridization reaction is carried out in the pH range of 5 to 9, the rate of hybridization is independent of pH.

(iv) Blocking DNA (or RNA), which is non-specific DNA (or RNA), to reduce non-specific hybridization by binding to molecules in the cytoplasm or nucleus that would otherwise bind probe or detection reagents. DNA used for this purpose is fragmented by physical or chemical means to an average of 100-200 base pair fragments. Commonly used blocking DNA includes calf thymus DNA and fish sperm DNA. Oligonucleotides such as Poly(C) and Poly(A) can also be used.

The hybridization solution may also contain the following optional components:

(i) Polymers, that are strongly hydrated in aqueous solution and prevent access to hydrated water by macromolecules, thereby increasing the probe concentration and consequently the hybridization rate. Examples include dextran sulphate, polyethylene glycol and similar polymers. Non-polymers such as phenol can also be used to increase the hybridization rate.

(ii) Detergents such as sodium dodecyl sulphate (SDS), CHAPS™, Triton-X100™, Brij35™ and Brij58™

which act as wetting agents and as permeabilizing agents to assist in probe penetration to the cytoplasm.

(iii) Chelating agents such as EDTA, citrate or similar agent to remove cations that can strongly stabilize duplex DNA.

(iv) Bovine serum albumin (BSA) or Denhardt's reagent (0.02% Ficoll, 0.02% polyvinyl pyrrolidone and 0.02% BSA) can also be included to reduce non-specific hybridization.

A hybridization solution suitable for this invention includes 30%-60%, preferably 50% formamide, 0.1x-6xSSC, preferably 2xSSC, 5%-10% (w/v), preferably 10% (w/v) dextran sulphate, 0.1μg-10μg/μl, preferably 1μg/μl fish sperm blocking DNA, 1-10ng/μl, preferably 5ng/μl of each labelled probe. Other hybridization solutions suitable for in situ hybridization can be used and are known to skilled in the art See eg., A.R. Leitch et al., In Situ Hybridization, Bios Scientific, (1994) Oxford, England; B.D. Hames et al., Nucleic Acid Hybridization: A Practical Approach, IRL Press, (1988) Oxford, England.

b. **Determination of hybridization conditions**

In general the hybridization conditions will depend on the type of hybrids to be formed (ie DNA:DNA or DNA:RNA or RNA:RNA) and whether the sequences are closely related or distantly related. Hybridization conditions suitable for this invention are those that promote the formation of well matched hybrids using conditions of high stringency.

(i) Genetic Probe size and concentration

Maximal hybridization rates are obtained with long probes since the rate of renaturation is proportional to the square root of the single-stranded probe length. However, for in situ hybridization, the probe must also be small enough to diffuse into the dense matrix of the cell. Nucleic acid probes suitable for this invention are 100bp-1000bp, preferably 200bp-400bp in length. Oligonucleotide probes suitable for this invention are preferably about 15bp-50bp in size.

The probe concentration affects the rate of the nucleation reaction which is the rate limiting step in hybridization and refers to the formation of the first few base pairs. Once nucleation occurs, adjacent base pairs will form to give a zippering effect. The higher the probe concentration, the higher the annealing rate, but at very high probe concentrations background signals will be generated. A probe concentration which increases the annealing rate without excessive background signal is preferred. Preferred probe concentrations are at about 1 to 10ng/µl, preferably about 5ng/µl.

(ii) Hybridization temperature

Hybridization depends on the ability of denatured nucleic acids to reanneal with complementary strands in a hybridization solution maintained at a temperature that is just below their melting point (T_m). The broad maximum rate for nucleic acid reannealing occurs from 20°C to 30°C below the T_m.

Hybridization buffers suitable for this invention contain the chaotropic agent formamide (at 50%-60% v/v) and employ hybridization temperatures for DNA:DNA and DNA:RNA hybrids of 30°C to 45°C, preferably 37°C to 42°C, and for RNA:RNA hybrids, temperatures of 50°C to 60°C, preferably 55°C.

c. Removing non-specifically bound genetic probe

The hybridization solution is removed and the cell suspension is washed to remove non-specifically bound probe. The wash buffer, washing time, temperature, and frequency of washes varies depending on the probe used. Removal of non-specifically bound probe is usually carried out under high stringency conditions at about 5°C to 25°C below the T_m of the perfectly matched duplex and at low salt concentrations of 0.01xSSC-2xSSC, preferably 0.1-1xSSC. Other wash buffers suitable for in situ hybridization are known to those skilled in the art. See eg., A.R. Leitch et al., In Situ Hybridization, Bio Scientific (1994) Oxford, England; B.D. Hames et al., In Nucleic Acid Hybridization: A Practical Approach, (1988) IRL Press, Oxford, England. Washing is usually carried out with several changes of wash buffer for 2-20 min each, preferably 5 min each wash. Cells are pelleted by centrifugation between washes. The total volume of wash buffer used should be 5-10 times the original volume of the hybridization solution.

Following in situ hybridization and washing, cells are concentrated by centrifugation at 100 x g to 4000 x g, preferably 400 x g, at 0°C to 25°C, preferably 4°C, for 1-60

min, preferably 15 minutes and then resuspended in an appropriate volume of PBS (see below).

B. Antibody Detecting Agents

In another embodiment of the invention, an antibody can be a detecting agent. An antibody is a protein that is produced by blood plasma cells in response to an antigen or a hapten associated with a suitable carrier. Antigens include proteins, peptides, polypeptides, carbohydrates, nucleic acids, amino acids, lipids, metabolites, and protein labels.

Antibodies are of two types; polyclonal antibodies that react with different parts of the same antigen molecule or monoclonal antibodies that are specific for a single antigenic determinant. Both types of antibodies are suitable for this invention. Methods for the preparation of both types of antibodies are known to those of skill in the art. See eg., Antibodies: A Laboratory Manual (Harlow E. et al., eds. 1988), Cold Spring Harbor Press, New York, USA; Monoclonal Antibodies: Principles and Practice (Goding, JW. ed. 1986) Academic Press, London, England.

Antibodies act as detecting agents by binding to a selected intracellular constituent in a target cell. Accordingly, as used in the present invention, the term "hybridization" and derivatives thereof, may be used to refer to the attachment of a detecting agent to an intracellular constituent, whether the detecting agent is an antibody, genetic probe or other agent which performs the function of a detecting agent as described herein. Antibody detecting

agents are preferably detectable by the below-described solid phase support system.

Prior to antibody hybridization, the mixed cell population can be incubated with a blocking solution to prevent non-specific hybridization of the antibody to non-specific sites. Blocking solutions are known in the art and include, for example, 100mM Tris/HCl at pH 7.5, 150mM NaCl or PBS or SSC containing a blocking reagent such as 0.5%-2% bovine serum albumin, 0.5%-5% normal serum or 0.5%-5% non-fat dry milk and may also contain a detergent such as 0.05%-0.2% Tween20™ or SDS or TritonX-100™.

Antibody detecting agents can be used individually or as mixture of several antibodies. The antibodies may be used at dilutions ranging from undiluted to 1:10,000. The dilution used is determined empirically for each antibody but typically polyclonal antibodies are used as dilutions of 1:10 to 1:100 whereas monoclonal antibodies are used at dilutions of 1:500 or greater. The antibodies are incubated with permeabilized cells for a period of 30 min to 24 hours, preferably 30min-3 hours at 0°C to 37°C, preferably 4°C. The antibody hybridization solution contains 100mM Tris/HCl; pH 7.5, 150mM NaCl or PBS or SSC and may contain a blocking reagent such as 0.5%-2% bovine serum albumin, 0.5%-5% normal serum or 0.5%-5% non-fat dry milk and may also contain a detergent such as 0.05%-0.2% Tween20™ or SDS or TritonX-100™.

Following antibody binding, the cells are washed 4-5 times for 2-20 min each, preferably 5 min each wash, using 100mM Tris/HCl; pH 7.5 or PBS or SSC containing 0.5%-5% normal

serum or 0.5%-2% bovine serum albumin. The washing buffer may also contain a detergent such as 0.05%-0.2% Tween20™ or SDS or TritonX-100™. Cells are pelleted by centrifugation between washes.

VI. Solid Phase Enrichment

Cells of the mixed cell population which are detected by hybridization of a detecting agent are concentrated using a solid phase support system (SPSS). Preferably, the solid phase support system of the invention concentrates a target cell by separating an enriched cell complex from non-complexed cells in a mixed population. An "enriched cell complex" refers to the combination of a target cell, a detecting agent and a solid phase support. Typically, an enriched cell complex includes an intracellular constituent hybridized to a detecting agent which is coupled to a solid phase support.

According to the invention, a solid phase support system includes a solid phase support which can couple to a detecting agent hybridized to an intracellular constituent. Hence, a solid phase support system may include a ligand or other component for coupling the detected target cell to the solid phase. Alternatively, a detecting agent may couple to the solid phase support directly. A solid phase support system (SPSS) may also include a mechanism for separating the enriched cell complex from other cells in a mixed cell population, for example, in the case of a magnetizable

particle solid phase support, a SPSS can include a magnetic field.

A. Solid Phase Support Systems

1. Solid Phase Supports

Solid phase supports suitable for the invention are known in the art and include, for example, magnetizable particles (see eg., Pourfarzaneth et al., The Use of Magnetizable Particles in Solid Phase Immunoassay, in Methods of Biochemical Analysis 28, 267-295 (D. Glick ed. 1981) John Wiley, New York), silica, agarose, glass, dextran, fibre supports, cellulose and synthetic polymers, and similar supports (see eg. Affinity Chromatography 12-145, (Lowe CR. et al., eds. 1974) Wiley and Sons, London, England).

According to the invention, a preferred solid phase support is a superparamagnetic particle. Methods for preparing superparamagnetic particles suitable for this invention are known to those skilled in the art. See eg., S. Miltenyi et al., Cytometry 11:231-238 (1990); E.V. Groman et al., Biotechniques, 156-160 (1985); J.T. Kemshead et al., Mol. Cell. Biochem 67:11-18 (1985); Pourfarzaneth et al., Methods of Biochemical Analysis 28, 267-295 (D. Glick ed. 1981), John Wiley, New York)

2. Ligands

A solid phase support system may include a ligand for coupling a detecting agent to the solid phase support. The ligand preferably exhibits specific binding affinity for the detecting agent. The ligand is typically immobilized by also

binding to the solid phase support. In general, the ligand may have a dissociation constant (K_d) for the detecting agent in the range of $K_d=10^{-4}$ to 10^{-8} M in free solution. Ligands such as streptavidin or avidin can be used to provide an extremely stable linkage ($K_d=10^{-15}$ M) with a biotin labelled detecting agent. The ligand can have chemically modifiable groups that allow it to be attached to the solid phase without destroying its binding activity. Chemically modifiable groups include amino, aldehyde, carboxyl, thiol, hydroxyl and mercurated bases. Where there is no information on the location of chemically modifiable groups in the ligand, a systematic trial and error approach is used to identify a modifiable group that does not destroy the binding activity of the ligand. The ligand can be a protein, peptide, amino acid, sugar, polynucleotide enzyme, coenzyme, cofactor, antibiotic, steroid, antibody, nucleic acid, hormone or vitamin. Examples of ligands include; antigen:antibody interactions where the ligand is an antibody which can couple to a solid phase support such as a synthetic polymer; glycoprotein:lectin interactions where the ligand is lectin which can couple to a synthetic polymer; receptor:ligand interactions where the ligand can couple to a synthetic polymer, for example, Sepharose™. In the above examples, the ligand can couple to the solid phase support through an amino group.

Methods for coupling ligands to solid supports using the above referred chemically modifiable groups are known in the art. See eg., Affinity Chromatography, (Lowe CR. et al., eds. 1974) Wiley and Sons, London, England).

When genetic probes are used as detecting agents, preferably, the ligand displays specific binding to the label incorporated into the genetic probe. For example, when the detecting agent is a genetic probe labelled with biotin, a component of the solid phase support system includes avidin or streptavidin. Alternatively, the ligand can be an antibody raised to the specific label that has been incorporated into the genetic probe in the labelling reaction.

When the detecting agent is an antibody, a component of the solid phase support system may include a secondary antibody (the ligand) directly coupled to the solid phase support which binds to the detecting agent (the primary antibody).

In one embodiment of the invention, the ligand may be coupled to a superparamagnetic particle (the solid phase support). Methods for coupling ligands to superparamagnetic particles are known in the art. (See eg., S. Miltenyi et al. Cytometry 11:231-238 (1990); E.V. Groman et al., Biotechniques, 156-160 (1985); J.T. Kemshead et al., Mol. Cell. Biochem 67:11-18 1985); Pourfarzaneth et al., Methods of Biochemical Analysis, 28, 267-295 (D. Glick ed. 1981), John Wiley, New York).

Once coupled to the solid phase support system, an enriched cell complex is formed. An enriched cell complex includes a target cell having a detecting agent attached to an intracellular constituent of the target cell which is coupled to the solid phase support.

However, in some arrangements, the ligand can be eliminated if the detecting agent is directly coupled to the solid phase support. For example, if the target intracellular constituent in the target cell is a protein and the detecting agent is an antibody raised to that protein, the antibody may be directly coupled to the solid support through chemical means. Alternatively, if the target constituent in the cell is a nucleic acid, the detecting agent may be a genetic probe that is directly coupled to the solid support through, for example, chemical means. Direct coupling of the detecting agent to the solid phase support may reduce the efficiency of hybridization to the intracellular constituent when using a genetic probe or antibody as a detecting agent because of the possibility of stearic hindrance of the solid phase.

B. Binding to the solid phase support

1. Non-magnetic solid phase supports

In one embodiment of the invention, non-magnetic solid phase supports may be used. These supports are most often packed into a chromatography column and equilibrated by passing through 10 volumes of a column buffer. The volume of solid support will depend on the number of total cells added to the column and the binding capacity of the solid support. Cell populations of 10^2-10^6 are separated with 2-5ml of solid phase support containing about 1mg/ml of a protein ligand such as an antibody or lectin. Column buffers are chosen according to the cell type to be separated and are most often buffered with Tris (Tris= Tris[hydroxymethyl]aminomethane) or HEPES (HEPES= N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and contain sodium azide(0.02% w/v) and a protective colloid

such as Ficoll70 (0.3% w/v), or human serum albumin (0.3%) or gelatin (0.25%). Separations are usually carried out at temperatures below 37°C.

Preferably, the mixed cell population is added to the column in a volume of 30% or less of the volume of the solid phase support. This percentage is not absolute but rather, is a recommendation to prevent overloading of the column which may reduce the efficiency of the enrichment. The cells are passed into the solid phase support and the buffer flow stopped. Cells are allowed to be in contact with the solid phase support preferably, for about 2-20min to form the enriched cell complex, the precise time of contact may vary, longer incubation times (ie. 15-20min) enhances efficient removal of a specific cell type whereas shorter times (ie. 2-10min) lessens the risk of contamination with other cells.

After incubation of the cells with the solid phase support, the column is washed. Washing is carried out using approximately 20 volumes of the column at a rate of 2-10ml/min buffer wash to remove non-specifically bound material prior to removing cells from the solid phase support (elution).

2. Magnetic solid phase supports

As previously discussed, solid phase supports suitable for the invention include magnetizable particles. Preferably, the magnetizable particle is a superparamagnetic particle. Superparamagnetic solid phase supports generally require no equilibration, however, superparamagnetic particles can be washed in buffer prior to use. The amount of

superparamagnetic particles used for cell enrichment depends on the cell type to be isolated and the total number of cells in the population.

Generally, superparamagnetic particles fall into two size ranges: >1μm diameter particles; and <150nm diameter particles. Particles >1μm in diameter require low gradient magnetic systems, for example, cobalt samarium permanent magnets. Particles <150nm in diameter require high gradient magnetic systems where magnetic steel wire mesh is packed into a column. When such columns are placed between the poles of a strong permanent magnet (0.6 Telsa), a very large field gradient is generated next to the wire and cells coated with <150nm particles are attracted to the wire.

a. Low gradient magnetic systems

Typically for superparamagnetic particles (>1μm), the ratio of particles to total cells ranges from 2:1 to 20:1 depending on the cell type, with a total cell number of 10^6-10^9 cells, in volumes ranging from 5μl (for 10^6 total cells) to 5.0 ml (for 10^9 total cells).

Buffers such as PBS are used and may contain sodium azide (0.01%) and bovine serum albumin (0.1%) or fetal calf serum(1.0%). Magnetic separations are typically carried out at temperatures below 37°C.

Superparamagnetic particles are combined with the cell population using the ratios described above and then incubated for 5-30min at 2°C-12°C with constant agitation. The ligand

which is coupled to the solid phase support system binds to the detecting agent which is hybridized to the selected intracellular constituent of the target cell to form an enriched cell complex. The mixture is then placed into a magnetic field. The magnetic field will attract the enriched cell complex. Non-target cells are separated by washing the column.

Washing in a low gradient magnetic system is carried out by aspirating the excess solution thilecells bound by the magnetic particles are kept on the wall of the tube. The tube is then removed from the magnet and the cells are resuspended in washing buffer (PBS containing 0.1% w/v bovine serum albumin). The washing process is repeated 4-5 times to remove non-target cells. The total volume of buffer used for the washes should be 5-10 times the volume in which the particle:cell complex was originally formed.

b. **High gradient magnetic systems**

Superparamagnetic particles of size <150nm are used at a higher ratio of to cells because of their very small size. Typically, ratios of 1000:1 or greater are preferred. Total cell numbers range from 10^6-10^{10}, with wire mesh areas of $10cm^2$ (for 10^6-10^7 cells) to $10cm^3$ (for 10^9-10^{10} cells) in volumes ranging from 0.5ml (for 10^6-10^7 cells) to 50ml (for 10^9-10^{10}).

Buffers described above for low gradient magnetic systems can be used. Magnetic separations are usually carried out at temperatures below 37°C.

Superparamagnetic particles are combined with the cell population, in the ratios described above, and then incubated for 5-30min at 2°C to 12°C with constant agitation to form the enriched cell complex.

The mixture is loaded onto a column containing wire mesh in the presence of a magnetic field and the sample is passed slowly through the column. The column is washed with 3-5 times the column volume with PBS containing 0.1% w/v bovine serum albumin. The flow of buffer is stopped, the column removed from the magnetic field and cells are back-flushed with buffer. The column is again placed in the magnetic field and the flow of buffer continued. Finally, the flow is stopped again, the column removed from the magnetic field and the "magnetic" cell fraction is collected from the column and concentrated by pelleting the cells at 400 x g. The cells are resuspended in about 100µl PBS.

C. <u>Removing target cells from the solid phase support</u>

Specific cells of the enriched cell complex may be removed from the solid phase support and used for further diagnostic, therapeutic or research purposes. Methods for eluting the concentrated specific cells to provide an enriched cell population are determined by the type of solid phase support used, the type of ligand coupled to the solid phase support and the type of coupling of the ligand to the solid phase support. Generally, elution agents are used to remove the concentrated target cells from the solid phase support system. Removal of the specific cells from the solid phase

support is a compromise between the harshness of the eluent needed for elution and the risk of denaturing or destroying the bioactivity of the eluted material. Elution agents include compounds that weaken ionic bonding, hydrogen bonding, covalent bonding, van der Waals interactions or electrostatic forces that maintain the ligand:detecting agent complex. Elution may invoke changes in pH changes, changes in ionic strength, changes in polarity of the eluant, deforming agents or electrophoretic desorption. Another common means of elution is affinity elution where the eluting agent competes for binding to the detecting agent, or for binding to the ligand. Elution can occur either by a concentration gradient of a single eluant or by pulse elution using several elution agents. (See eg., Affinity Chromatography, 52-70, C.R. et al., eds. 1974), Wiley and Sons, London, England). In cases where the solid phase support does not need to be regenerated, the ligand can be destroyed by physical, chemical or enzymatic means to release the target cell.

1. Removal of magnetic particles from cells

In one embodiment of this invention, the solid phase support is a superparamagnetic particle. Particles in the <150nm range are biodegradable, are not visible by light microscopy or flow cytometry and do not interfere with either the post enrichment identification system or signal-generating system (see below), therefore it is unnecessary to carry out removal of the magnetic particle. If removal is required, incubation overnight at 37°C can be used to remove the particles from the cells. Particles in the >1μm range can be

removed from the cells by heating in 95% formamide at temperatures of at least 65°C for at least 2 min, or by boiling in the presence of 0.1% SDS for 5 min, or by incubation overnight at 37°C. When using the biotin:avidin (or streptavidin) identification system (see below) the use of biotin-nucleotide analogues that incorporate a cleavable linker arm into the biotin molecule can be used to dissociate the detecting agent from the superparamagnetic particle.

VII. Target Cell Identification Post Solid Phase Enrichment

Target cell enrichment may not yield a completely pure target cell population. Accordingly, once the enriched cells are eluted from the solid phase support, preferably, target cells are identified using the below described identification system and signal generating system.

As used herein, an "identification system" identifies the target cell using an "identifying agent" coupled directly or indirectly to a signal generating system. Generally, an identifying agent identifies a target cell by binding to a detecting agent (hybridized to an intracellular constituent prior to enrichment) or by binding directly to an intracellular or extracellular constituent of the target cell. Hence, an identifying agent includes all compounds which were previously described as a detecting agent.

If an identifying agent, such as a nucleic acid or antibody, binds directly to an intracellular constituent, it

may bind to the same, or a different intracellular constituent than that bound by the detecting agent. The identification system may also include a label which is incorporated into the detecting agent, intracellular constituent or extracellular constituent, that is bound by the identifying agent. Alternatively, an identification system may be the detecting agent which hybridized to an intracellular constituent prior to solid phase concentration.

A signal generating system provides visualization of a target cell. The signal generating system may be incorporated into an identifying agent, or incorporated into a compound which binds to an identifying agent. If the identifying agent is the detecting agent, the signal generating system may or may not be incorporated into the detecting agent prior to solid phase enrichment.

The methods for identification and visualization of enriched target cells will depend on the type of identification system used. If, for example, the identifying agent is to identify a target cell by binding to a detecting agent which is a nucleic acid with a label such as digoxigenin, 2-acetylaminofluorence, a sulphone group, mercury/trinitrophenol, or biotin then an immunocytochemical identifying agent may be used with a fluorochrome, enzyme-generated precipitate or metal signal generating system. If the identifying agent is to identify a detecting agent with a biotin label, an avidin (or streptavidin) identifying agent may be used with a fluorochrome, enzyme-generated precipitate or metal signal-generating system. If the detecting agent

used was a nucleic acid probe with a fluorochrome-nucleotide incorporated, no further identification or signal generating system may be needed. Visualization can be direct, by fluorescence. Alternatively, identification can be indirect by using an immunocytochemical identifying agent and a fluorochrome, enzyme-generated precipitate or metal signal-generating.

A. Identification Systems

1. Immunocytochemical Identifying Agents

According to the invention, immunocytochemical identification and visualization can be carried out in a one stage or two stage process. In the one stage process the signal generating system is coupled to an antibody which binds to a detecting agent, a label incorporated into a detecting agent, an intracellular constituent or an extracellular constituent. Identification occurs by the antibody (identifying agent), with the coupled signal generating system, binding to the antigen.

In the two stage process, identification and visualization includes a primary and secondary antibody. The primary antibody binds to an antigen, such as a detecting agent, a label incorporated into a detecting agent, an intracellular constituent or an extracellular constituent. The secondary antibody which carries the signal generating system binds to the primary antibody. A two stage detection system is preferred because several secondary antibodies

carrying the signal-generating system can bind to the primary antibody which provides for significant amplification of the signal thereby enhancing the sensitivity of visualization.

2. **Biotin:avidin (or streptavidin) identification system**

A biotin:avidin (or streptavidin) identification system uses a biotin label. The biotin label may be incorporated into the detecting agent prior to solid phase enrichment or the biotin may be incorporated into an identifying agent. The avidin (or streptavidin) is conjugated to a signal-generating system. The avidin (or streptavidin) then binds to biotin. Amplification of this biotin-avidin complex can be obtained by adding a biotinylated anti-avidin (or streptavidin) antibody followed by another layer of avidin (or streptavidin) conjugated to a signal-generating system.

Using a biotin label, either immunocytochemical or biotin:avidin (or streptavidin) systems are suitable for identifying a target cell. Methods for employing immunocytochemical identification systems and the biotin:avidin (or streptavidin) identification systems are known in the art See eg., Leitch, et al., In Situ Hybridization, Bios Scientific (1994), Oxford, England; B.D. Hames, et al., In Nucleic Acid Hybridization: A Practical Approach, (1988) IRL Press, Oxford, England.

B. Signal Generating Systems

According to the invention, visualization of a target cell identified by an identifying agent may be accomplished through use of a signal-generating system. Signal generating systems suitable for the invention include any compound which can couple to an identifying agent for visualization of a target cell. Preferred signal generating systems include, for example, fluorochromes, enzyme-generated precipitate or metals.

1. Fluorochromes

A fluorochrome is a chemical compound that emits fluorescence at a specific emission wavelength when excited by light of the appropriate excitation wavelength. By incorporating different fluorochrome-nucleotides into an identifying agent, such as a genetic probe, it is possible to carry out in situ hybridization with multiple genetic probes and to directly identify the presence of multiple hybridized probes in a single cell by using the light of the appropriate excitation wavelength for each different fluorochrome. Fluorochromes can also be attached to avidin (or streptavidin), or to antibodies, which allows them to be used in the immunocytochemical identification systems and biotin-avidin (or streptavidin) identification systems described above.

2. **Enzyme-generated precipitates**

Two commonly used enzymes for immunocytochemistry are alkaline phosphatase and horse radish peroxidase. When conjugated to an antibody or to avidin (or streptavidin), the enzymes allow visualization by catalyzing the localized precipitation of a colored product at the site where the probe has bound, following addition of the appropriate substrate.

If present, endogenous alkaline phosphatases and peroxidases are preferably inactivated prior to immunocytochemistry. Endogenous alkaline phosphatases found in placental and intestinal tissue may be inactivated by, for example, the addition of levamisole or 20% acetic acid. Endogenous peroxidases, which may be found in various cell types in the blood, can be inactivated by borohydride, periodate or phenyl hydrazine.

3. **Metals**

Colloidal gold conjugated to an antibody or to avidin (or streptavidin) can be used with both of the above types of identification systems. The metal allows the target cell, bound to an identifying agent, to be visualized either by light microscopy or electron microscopy.

Methods for employing the signal-generating systems described above, are known in the art and are discussed, for example, in A.R. Leitch, et al., In Situ Hybridization, Bios Scientific (1994), Oxford, England; B.D. Hames et al., Nucleic

Acid Hybridization: A Practical Approach (1988), IRL Press, Oxford, England).

VIII. Evaluation of Enriched Cells

Target cells that have been enriched and identified according to the invention are suitable for further analysis. The types of analysis fall into two broad categories: analysis of intact cells and analysis of intracellular components.

A. Analysis of Enriched Intact Cells

Following enrichment and identification, cells may be deposited on slides and visualized either with a light microscope when employing enzyme-generated precipitate or metal signal generating systems. When identifying agents are used that are either directly or indirectly labelled with a fluorescent label, the results can be visualized on a fluorescence microscope, employing a wavelength emission filter appropriate for the particular fluorochrome to be visualized. Cells may also be automatically analyzed using a computer-controlled fluorescence-based image analysis system.

In general, the target cell of interest may express several intracellular specific nucleic acids or protein products that can be used to provide additional means for enriching a particular cell type of interest. As stated earlier, the method of the invention can also be combined with known methods of extracellular hybridization. Accordingly,

the enriched cell population can be subjected to further rounds of in situ hybridization and solid phase enrichment using detecting agents for other intracellular or extracellular constituents.

The enriched cells can also be subjected to further in situ hybridization analysis or to fluorescence activated cell sorting using other detecting agents specific for a selected intracellular constituent. For example, when fetal cells have been enriched from maternal blood, the use of labelled chromosome-specific probes and in situ hybridization can provide information on the chromosome complement of the fetus and in doing so identify chromosomal aneuploidy.

Mixed cell populations depleted of a particular cell type, can be reused to isolate other specific cell types in the population. This can be achieved by using another round of in situ hybridization and solid phase enrichment using detecting agents specific for a selected intracellular constituent of other cell types in the cell population.

B. **Biochemical and Genetic Analysis**

Cells that have been concentrated by in situ hybridization and solid phase enrichment are suitable for biochemical and genetic analysis. For example, nucleic acids extracted from the cells can be analyzed by molecular biological techniques, such as Northern and Southern blotting analysis, to identify specific nucleic acid sequences that are present in the enriched cell population. The technique of PCR

can also be used to identify specific nucleic acid sequences in enriched cell populations. Episomal elements such as eukaryotic or bacterial plasmids or viral nucleic acids can also be isolated from the enriched cells and reintroduced into an appropriate host for further analysis. Proteins extracted from the enriched cells can be analyzed by Western blotting.

One skilled in the art will readily recognize the potential clinical uses for the method and enriched cell complex of the present invention in the diagnosis, prognosis and therapy of disease and for further research purposes. The ability to reliably detect specific cells present in low concentration may, for example, provide a safe and cost effective means for diagnosis of fetal genetic abnormalities by enrichment of fetal cells from maternal blood (See eg. M. Adinolfi, Prenatal Diagnosis 15:889-896 (1995)). The method may also provide for the early diagnosis of oncogenic disease based on the presence of target cells in blood, lymph, bone marrow or other body fluid or tissue, as well as provide screening for HIV, or other infectious viral, bacterial or parasitic disease. The invention may also provide a non-invasive method for detecting an individual's genetic predisposition to various conditions, for example, heart disease, prostate cancer, breast cancer, leukemia and other conditions where genetic predisposition may be a factor. The method may also provide for detecting cells shed from tumors, carcinomas, sarcomas and melonamas into lymph fluid and into the circulatory system and in so doing, may provide a prediction for the likelihood of metastasis.

The ease and safety of the method further provides for minimally invasive evaluation of therapeutic efficacy of, for example, anti-bacterial, anti-viral, and anti-cancer treatments. In addition, because target cell enrichment also causes target cell depletion of a mixed cell population, it is foreseeable that the present invention may lead to new methods for treatment of diseases such as leukemia, AIDS, blood-borne parasitic diseases and similar diseases which may be ameliorated through depletion of selected cells.

The inventor also foresees the use of the present invention in combination with other methods, such as extracellular based solid phase enrichment, fluorescence activated cell sorting, and molecular biological methods.

IX. Solid Phase Enrichment Kit

The invention further provides a portable method for enrichment of at least one specific cell from a mixed cell population using a solid phase support system. A portable enrichment system may be included into a kit which may be assembled and packaged for use in enriching one or more specific cells. According to this embodiment of the invention, a kit may include, at least, a fixing agent, a permeabilizing agent, a detecting agent and a solid phase support system as described previously. The solid phase support system may include a portable solid phase support, for example, magnetizable particles, silica, agarose, dextran, fibre supports, cellulose, synthetic polymers and similar

supports. The kit may further include an identifying agent and a signal generating system.

The fixing agent and permeabilizing agent included with a kit may be any of those agents previously described. In addition, a single detecting agent may be included for identification of a single specific cell. Alternatively, multiple detecting agents may be included for detecting multiple specific cell types or detecting a single specific cell type based on the presence of multiple intracellular constituents.

The following Examples are designed to teach those skilled in the art of how to practice the invention. They are not intended to define or limit the scope of the invention in any way.

EXAMPLES

Example 1 Enrichment of fetal cells using intracellular messenger RNA (mRNA) gene products from maternal blood in a model system.

Enrichment of fetal cells from maternal blood was performed in a model system where known numbers of fetal trophoblast cells (called syncytiotrophoblast sprouts) were prepared and then added to maternal blood and then recovered using an enrichment procedure. (C.S. Hawes, et al., From Fertilisation to Fetus: Detection of Geno-Pheno-Type Diversities, 219-223, H. Zakut ed., (1994), Monduzzi Editore, Bologna, Italy).

Three detecting agents were compared. A 435bp partial cDNA fragment encoding the 3-β-hydroxysteroiddehydrogenase (3βHSD) gene was excised from a plasmid cDNA clone pCMV5H3β-HSD (Lorence et al., Endocrinology 126:2493-2498 (1990)) by restriction digestion with EcoRI/BamHI. A 1.2kb partial cDNA fragment encoding the human placental lactogen hormone (hPL) gene was excised from a plasmid cDNA clone pN202(18) (S. Latham et al., Prenatal Diagnosis, 16:813-821 (1995)) by restriction digestion with EcoRI. A 0.9kb partial cDNA fragment encoding the PSG1 gene was excised from a plasmid cDNA clone pSP10.9 (S. Latham and B. Kalionis unpublished data) by restriction digestion with EcoRI. Each DNA fragment was labelled separately with digoxigenin-11-dUTP, using the random-primed labelling kit from Boehringer Mannheim (DIG-High Prime kit, 1995 Catalogue No. 1-585-606) and following the manufacturers instructions included in the kit.

In situ hybridization experiments with genetic probes to the 3βHSD gene, hPL gene and PSG1 genes have shown these genetic probes to be specific for syncytiotrophoblast sprouts and they do not show significant cross-hybridization with other cells in blood (H. Suskin and B. Kalionis, unpublished data).

First trimester placental tissue was collected into saline. Excess blood was rinsed off with saline and a suspension of syncytiotrophoblast sprouts was prepared by vigorously shaking the freshly obtained tissue in 20-30 ml saline at room temperature. This caused the syncytiotrophoblast sprouts to be shed from the chorionic

villi. 50µl aliquots of the cell suspension were spotted onto slides, air-dried and stained with haematoxylin. The total number of syncytiotrophoblast sprouts in each aliquot was counted microscopically to determine an average number per aliquot. For example, the counts obtained in a typical series of five aliquots were 68, 70, 71, 71 & 72. From this, the volume of suspension containing approximately 50 syncytiotrophoblast sprouts was calculated (eg. 35 µl).

50 syncytiotrophoblast sprouts (see above) were added to 20 ml of human peripheral blood cells in 10ml tubes and then incubated with lysis buffer (0.1M NH_4Cl, 15mM $NaHCO_3$, 0.1mM Na_2EDTA) to lyse the red blood cells, centrifuged at 400 x g for 5 min, incubated a second time in lysis buffer and then washed with 50 ml cold saline. The white blood cells were centrifuged at 400 x g for 10 minutes in a clinical centrifuge (yielding a total of about 10^8 cells). The cells were fixed by resuspending in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Centrifugation was repeated at 400 x g to pellet the cells. The excess fixative was withdrawn by aspiration. The cell pellet was washed in PBS for 5 min and again centrifuged to pellet the cells. The PBS was removed by aspiration and the cells washed once more.

The cells were then permeabilized with Proteinase K at 100 µg/ml for 10 min at 37°C. Permeabilization was stopped with 0.2% (w/v) glycine in PBS for 2 min at room temperature. The cells were pelleted at 400 x g and excess glycine removed. The cells were then post-fixed in 4% paraformaldehyde as described above.

The cells were washed twice with PBS as described above with saline and the cell pellet was resuspended in hybridization buffer containing one or more of the genetic probes. The hybridization buffer contained 60% formamide, 2xSSC, 25mM NaH_2PO_4 pH 7.4, 5% dextran sulphate, 250 µg/ml sonicated, denatured salmon sperm DNA and a detection agent comprising one or more digoxigenin-labelled hybridization genetic probes at a concentration of 5ng/µl. Prior to resuspending the cells, the labelled genetic probe, in hybridization buffer, was denatured by incubating the mixture at 80°C for 10 min followed by snap chilling on ice for 5 min. Hybridization was carried out for 16 hours at 37°C. Cells were pelleted at 400 x g in a clinical centrifuge and the excess hybridization solution was removed. The cells were washed twice in 0.5XSSC at room temperature for 5 min and then washed a third time in 0.5xSSC at 37°C for 10min. The cells were again pelleted and washed in PBS for 5min.

Specific cells were then concentrated. A one in 100 dilution of a mouse anti-digoxigenin antibody (approx. 0.4 µg) (1995 Cat No. 1-333-062, Boehringer Mannheim, Germany) was added to the cells. The cells were incubated in the presence of the antibody for 2 to 3 hours at 37°C. The cells were then pelleted and excess antibody removed by aspiration and the cells washed twice in PBS as described above. The cell pellet was resuspended in 20 µl of Rat-anti-mouse IgG1-Microbeads (1992 Cat No. 271-01, Miltenyi Biotec Gmbh, Germany) and incubated at 4°C for 15 min. Magnetic columns (1992 Cat No. 211-02, Type A2, Miltenyi Biotec Gmbh, Germany) were pretreated by passing a mixture of ethanol/ethylsulfate/

isopropyl alcohol/siloxane through the column and then rinsing the column with PBS. The column was then placed into a strong magnetic field (1992 Cat No. 231-02, MACS Separator, Miltenyi Biotec Gmbh, Germany) and the magnetic bead:cell suspension was loaded onto the column, then washed with 5 ml of PBS/0.01% sodium azide/1%BSA buffer. The column was removed from the magnetic field and backwashed with 2 ml of the PBS/0.01% sodium azide/1%BSA buffer to dislodge any cells that were non-specifically bound to the column. The column was placed back into the magnetic field and the cells allowed to migrate back onto the wire-mesh. The washing and backwashing step was repeated four times.

The concentrated cells retained by the magnet after washing and backwashing were eluted by removing the column from the magnet and passing 10 ml of PBS buffer through the column. This elution was repeated once more. The eluted cells were then centrifuged at 400 x g for 5 min to pellet the cells.

The enriched cells were then identified and visualized. Pelleted cells were resuspended in approximately 50 µl of PBS buffer and then deposited onto a microscope slide and air dried. Cells on the slides were incubated in 20% normal sheep serum for 30 min at 20°C to block non-specific binding of the secondary antibody. Slides were briefly rinsed in PBS. A labelled secondary antibody, anti-digoxigenin-rhodamine Fab fragments from sheep (1995 Cat No. 1-207-750, Boehringer Mannheim, Germany), was applied to the cells at a dilution of 1:10 and incubated for 60 min at 20°C for target cell

identification and visualization. The slides were washed twice in PBS containing 0.1% v/v Nonidet P40 (Boehringer Mannheim, Germany). Anti-fade mountant (90% v/v glycerol 0.1% v/v p-phenylenediamine) was added to the cell samples and then a coverslip was applied. Cells were then detected with fluorescence microscopy using a 615nm emission wavelength filter and the total number of fluorescent cells determined. The entire sample was scanned under the microscope and the fluorescent syncytiotrophoblast sprouts counted.

TABLE 1 **Recovery of Syncytiotrophoblast Sprouts in a Model System**

Genetic Probe(s)[1]	Number of Sprouts Added[2]	% Recovery of Sprouts[3]
3βHSD	50	38,46
hPL	50	36,48
PSG1	50	44
3βHSD, hPL	50	68
3βHSD, hPL, PSG1	50	78
no genetic probe added	50	2

1. Genetic probe or probes used to recover syncytiotrophoblast sprouts;

2. Number of syncytiotrophoblast sprouts added; and

3. The percentage recovery of syncytiotrophoblast sprouts following in situ hybridization with the genetic probe(s) and solid phase enrichment. Each number represents percentage recovery per individual sample. The percentage recovery of contaminating white blood cells (WBC) was calculated from the number recovered in the magnetic activated cell sorting (MACS) eluate compared to the total WBC count of the sample (average 10^7 cells/ml) and was <0.001% in all cases (data not shown).

Table 1 shows that syncytiotrophoblast sprouts were recovered from a mixed population of cells in maternal blood using genetic probes as detecting agents and a superparamagnetic particle as solid phase support for enrichment. The efficiency of recovery of syncytiotrophoblast sprouts was improved by the use of multiple genetic probes.

Example 2 Enrichment of 5T33 myeloma cells using an intracellular messenger RNA (mRNA) for the IgH gene in a model system.

Myelomas are a tumor of cells that are derived from the hematopoietic tissue of bone marrow. Myeloma tumors are clonal in origin and secrete large amounts of a single species of antibody. In vitro cultured cell lines have been established from 5T33 myeloma tumors. See L.S. Manning et al., Br. J. Cancer 66:1088-1093 (1992). In myeloma cell lines, the immunoglobulin heavy chain (IgH) gene is expressed at high levels. PCR primers were used to specifically amplify a segment of the IgH gene to be used as the genetic probe. The PCR primeers used for amplification were FR1 5'-(GC) AGGT(CG)(AC)A(AG)CTGCAG(CG)AGTCT-3' (SEQ ID NO:1); and FR4 5'-GGAGACTCTGAGAGTGGTG-3' (SEQ ID NO:2).

The IgH PCR fragment was labelled with digoxigenin-11-dUTP, using the random-primed labelling kit from Boehringer Mannheim (DIG-High Prime kit, 1995 Catalogue No. 1-585-606) and following the manufacturers instructions included in the kit. In situ hybridization experiments with the IgH genetic probe have shown this probe to be specific for 5T33 myeloma

cells and the IgH genetic probe does not significantly cross-hybridize with other cell types in human maternal peripheral blood (H. Suskin and B. Kalionis, unpublished data). As a control, a genetic probe to the 3βHSD gene was used. Genetic probes to the 3βHSD gene do not show specific cross-hybridization with cells in human maternal peripheral blood or with 5T33 myeloma cells (H. Suskin and B. Kalionis, unpublished data). A genetic probe for the 3βHSD gene was prepared exactly as described in Example 1 above.

5T33 myeloma cell culture lines were grown and harvested using methods known in the art. (See eg., Culture of Animal Cells: A Manual of Basic Technique (R.I., Freshney ed. 1987), Wiley-Lis, New York, USA).

Approximately 1000 5T33 myeloma cells were added to 20 ml of human maternal peripheral blood cells and then incubated with lysis buffer (0.1M NH_4Cl, 15mM $NaHCO_3$, 0.1mM Na_2EDTA) to lyse the red blood cells, centrifuged at 400 x g for 5 min, incubated a second time in lysis buffer and then washed with 50 ml cold saline. The white blood cells were centrifuged at 400 x g for 10 minutes in a clinical centrifuge (yielding a total of about 10^8 cells). The cells were fixed by resuspending in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Centrifugation was repeated at 400 x g to pellet the cells. The excess fixative was withdrawn by aspiration. The cell pellet was washed in PBS and again centrifuged to pellet the cells. The PBS was removed by aspiration and the cells washed once more.

The cells were then permeabilized with Proteinase K at 100 µg/ml for 10 min at 37°C. Permeabilization was stopped with 0.2% (w/v) glycine in PBS for 2 min at room temperature. The cells were then post-fixed in 4% paraformaldehyde as described above. The cells were washed two more times with PBS and pelleted as described above.

Cells were resuspended in hybridization buffer containing 60% formamide, 2xSSC, 25mM NaH_2PO_4 pH 7.4, 5% dextran sulphate, 250 µg/ml sonicated, denatured salmon sperm DNA and the detection agent comprising a digoxigenin-labelled hybridization genetic probe at a concentration of 5ng/µl. Prior to resuspending the cells, the labelled genetic probe, in hybridization buffer, was denatured by incubating the mixture at 80°C for 10 min followed by snap chilling on ice for 5 min. Hybridization was carried out for 16 hours at 37°C. Cells were pelleted at 400 x g in a clinical centrifuge and excess hybridization solution was removed. The cells were washed twice in 0.5XSSC twice at room temperature for 5 min and then washed a third time in 0.5xSSC at 37°C for 10min. The cells were again pelleted and washed in PBS for 5min.

Specific cells were then concentrated. A one in 100 dilution of a mouse anti-digoxigenin antibody (approx. 0.4 µg) (1995 Cat No. 1-333-062, Boehringer Mannheim, Germany) was added to the cells. The cells were incubated in the presence of the antibody for 2 to 3 hours at 37°C. The cells were then pelleted and excess antibody removed by aspiration and the cells washed twice in PBS as described above. The cell pellet was resuspended in 20 µl of Rat-anti-mouse IgG1-Microbeads

(1992 Cat No. 271-01, Miltenyi Biotec Gmbh, Germany) and incubated at 4°C for 15 min. Magnetic columns (1992 Cat No. 211-02, Type A2, Miltenyi Biotec Gmbh, Germany) were pretreated by passing a mixture of ethanol/ethylsulfate/ isopropyl alcohol/siloxane through the column and then rinsing the column with PBS. The column was then placed into a strong magnetic field (1992 Cat No. 231-02, MACS Separator, Miltenyi Biotec Gmbh, Germany) and the magnetic bead:cell suspension was loaded onto the column then washed with 5 ml of PBS/0.01% sodium azide/1%BSA buffer. The column was removed from the magnetic field and backwashed with 2 ml of the PBS/0.01% sodium azide/1%BSA buffer to dislodge any cells that were non-specifically bound to the column. The column was placed back into the magnetic field and the cells allowed to migrate back onto the wire-mesh. The washing and backwashing step was repeated four times. Cells retained by the magnet after washing and backwashing were eluted by removing the column from the magnet and passing 10 ml of PBS buffer through the column. This elution was repeated once more. The eluted cells were then centrifuged at 400 x g for 5 min to pellet the cells.

The enriched cells were then identified and visualized. Pelleted cells were resuspended in approximately 50 µl of PBS buffer and then deposited onto a microscope slide and air dried. Cells on the slides were incubated in 20% normal sheep serum for 30 min at 20°C to block non-specific binding of the secondary antibody. Slides were briefly rinsed in PBS. A labelled secondary antibody, anti-digoxigenin-rhodamine Fab fragments from sheep (1995 Cat No. 1-207-750, Boehringer

Mannheim, Germany), was applied to the cells at a dilution of 1:10 and incubated for 60 min at 20°C. The slides were washed twice in PBS containing 0.1% v/v Nonidet P40 (Boehringer Mannheim, Germany). Anti-fade mountant (90% v/v glycerol 0.1% v/v p-phenylenediamine) was added to the cell samples and then a coverslip was applied. Cells were then detected with fluorescence microscopy using a 615nm emission wavelength filter and the total number of fluorescent cells determined.

TABLE 2 **Recovery of 5T33 Myeloma Cells in a Model System**

Genetic Probe(s)[1]	Number of 5T33 Myeloma Cells Added[2]	% Recovery of 5T33 Myeloma Cells[3]
IgH	1000	87,85,93
IgH	0	0.06,0.07
3βHSD	1000	0.04

1. Genetic probe or probes used to recover 5T33 myeloma cells

2. Number of 5T33 myeloma cells added

3. The percentage recovery of fluorescent 5T33 myeloma cells following in situ hybridization with the genetic probe and solid phase enrichment. Each number represents percentage recovery per individual sample. The percentage recovery of contaminating white blood cells (WBC) was calculated from the number recovered in the MACS eluate compared to the total WBC count of the sample (average 10^7 cells/ml) and was <0.001% in all cases (data not shown).

Table 2 shows that 5T33 myeloma cells were recovered from a mixed population of cells in maternal blood using a genetic probe as a detecting agent and a superparamagnetic particle as the solid phase support for enrichment. Recovery of cells was dependent on the addition of 5T33 myeloma cells to maternal blood. A 3βHSD genetic probe, which is not expressed either

in maternal cells or 5T33 myeloma cells, also gives very low recovery of cells indicating that recovery of cells was dependent on the addition of a specific genetic probe.

EXAMPLE 3 **In situ PCR amplification in liquid to amplify intracellular nucleic acid target sequences prior to enrichment using a model system.**

The human cytotrophoblast cell line, HTR8 (C.H. Graham et al., Experimental Cell Research, 206:204-211 (1993)) expresses the transcription factor gene Dlx-4 (L. Quinn and B. Kalionis, Gene, in press (1997)). HTR8 cells are grown in culture and harvested (C.H. Graham et al., Experimental Cell Research, 206:204-211 (1993)). Cells (approx. total of 10^5-10^6 cells) are then fixed by resuspending in 4% paraformaldehyde in PBS for 10 minutes at room temperature. The cells are centrifuged at 400 x g to pellet the cells. Excess fixative is withdrawn by aspiration and the cell pellet is washed in PBS for 5 min and again centrifuged to pellet the cells.

The cells are then permeabilized by treatment with Proteinase K at 100 µg/ml for 10 min at 37°C. Permeabilization is stopped with 0.2% (w/v) glycine in PBS for 2 min at room temperature. The cells are pelleted at 400 x g and excess glycine is removed. The cells are then post-fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Cells are pelleted at 400 x g and excess fixative is withdrawn by aspiration. The cell pellet is washed in PBS for 5 min and again centrifuged to pellet the cells.

Cells are resuspended in 100µl of buffer containing 50mM KCl, 20mM Tris-HCl (pH 8.4), 2.5mM $MgCl_2$, 0.1mg/ml bovine serum albumin, 1mM each dNTP, RNasin inhibitor (Promega Corporation, USA) at 1 unit/µl, 100pmol random hexamer oligonucleotides and 200 units of MoMuLV (or AMV) reverse transcriptase. The reaction is incubated at room temperature for 10 min and then at 37°C for 60 min. The reaction is terminated by heating at 95°C for 5 min. The cells are then pelleted at 400 x g, and excess solution is removed by aspiration. Cells are resuspended in 100µl of buffer containing 10mM Tris-HCl (pH 8.4), 1.5mM $MgCl_2$, 50mM KCl, 200µM each of dGTP, dCTP, dATP, 190µM dTTP, 10µM digoxigenin-11-dUTP, 1-2 units Taq polymerase, 100µg/ml gelatin and 0.25µM each of Dlx-4 specific 31ks primer (5'-AGTCTTCCGGGTGGAGC-3') (SEQ ID NO:3) and Dlx-4 specific 31sk primer (5'-GTCACTATCAGCGCTGC-3')(SEQ ID NO:4)(L. Quinn and B. Kalionis, Gene, in press (1997). The sample is overlayed with 75µl of mineral oil and the temperature raised to 95°C for 5 min, to denature nucleic acids in the cells. The cells are then subjected to 30 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1.5 min. Cycling is concluded with a final extension at 72°C for 10 min. The reaction is terminated by chilling to 4°C and addition of EDTA to 10 mM. The cells are then pelleted at 400 x g and resuspended in 4% paraformaldehyde in PBS and incubated for 10 minutes at room temperature to fix the cells.

Following amplification, approximately 1000 HTR8 cells are added to 20 ml of human peripheral blood cells, incubated with lysis buffer (0.1M NH_4Cl, 15mM $NaHCO_3$, 0.1mM Na_2EDTA) to

lyse the red blood cells, centrifuged at 400 x g for 5 min, incubated a second time in lysis buffer and then washed with 50 ml cold saline. The cells are centrifuged at 400 x g for 10 minutes in a clinical centrifuge (yielding a total of about 10^8 cells). The cells are fixed by resuspending in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Centrifugation is repeated at 400 x g to pellet the cells. The excess fixative is withdrawn by aspiration. The cell pellet is washed in PBS and again centrifuged to pellet the cells. The excess PBS is removed by aspiration and the cells are washed once more.

The cells are then permeabilized by treatment with Proteinase K at 10-100 µg/ml for 10 min at 37°C. Permeabilization is stopped with 0.2% (w/v) glycine in PBS for 2 min at room temperature. The cells are then post-fixed in 4% paraformaldehyde as described above. The cells are washed two more times with PBS and pelleted as described above.

Target cells are then concentrated. A one in 100 dilution of a mouse anti-digoxigenin antibody (approx. 0.4 µg) (1995 Cat No. 1-333-062, Boehringer Mannheim, Germany) is added to the cells. The cells are incubated in the presence of the antibody for 2 to 3 hours at 37°C. The cells are then pelleted and excess antibody is removed by aspiration and the cells are washed twice in PBS as described above. The cell pellet is resuspended in 20 µl of Rat-anti-mouse IgG1-Microbeads (1992 Cat No. 271-01, Miltenyi Biotec Gmbh, Germany) and incubated at 4°C for 15 min. Magnetic columns (1992 Cat No. 211-02, Type A2, Miltenyi Biotec Gmbh, Germany)

are pretreated by passing a mixture of ethanol/ethylsulfate/isopropyl alcohol/siloxane through the column and then rinsing the column with PBS. The column is then placed into a strong magnetic field (1992 Cat No. 231-02, MACS Separator, Miltenyi Biotec Gmbh, Germany) and the magnetic bead:cell suspension is loaded onto the column and then washed with 5 ml of PBS/0.01% sodium azide/1%BSA buffer. The column is removed from the magnetic field and backwashed with 2 ml of the PBS/0.01% sodium azide/1%BSA buffer to dislodge any cells that non-specifically bind to the column. The column is placed back into the magnetic field and the cells allowed to migrate back onto the wire-mesh. The washing and backwashing step is repeated four times. Cells retained by the magnet after washing and backwashing are eluted by removing the column from the magnet and then passing 10 ml of buffer through the column. This elution is repeated once more. The eluted cells are then centrifuged at 400 x g for 5 min to pellet the cells.

Pelleted cells are resuspended in approximately 50 µl of PBS buffer and then deposited onto a microscope slide and air dried. Cells on the slides are incubated in 20% normal sheep serum for 30 min at 20°C to block non-specific binding of the secondary antibody. Slides are briefly rinsed in PBS. A labelled secondary antibody, anti-digoxigenin-rhodamine Fab fragments from sheep, (1995 Cat No. 1-207-750, Boehringer Mannheim, Germany) is applied to the cells at a dilution of 1:10 and incubated for 60 min at 20°C. The slides are washed twice in PBS containing 0.1% v/v Nonidet P40 (Boehringer Mannheim, Germany). Anti-fade mountant (90% v/v glycerol 0.1%

v/v p-phenylenediamine) is added to the cell samples and a coverslip applied. Cells are then detected with fluorescence microscopy using a 615nm emission wavelength filter and the total number of fluorescent cells is determined.

Example 4 **Enrichment of cells using an intracellular messenger RNA (mRNA) expressed in prostate cells, in a model system and from patient samples.**

LNCaP cells (American Type Culture Collection CC CRL-1740 LNCaP.FGC Metastatic prostate adenocarcinoma, human) are an in vitro cultured cell line, originally derived from cells isolated from a needle aspiration biopsy of the supraventricular lymph node, from a patient with metastatic prostate carcinoma (Gibaz, Z. et al., Cancer Genet. Cytogenet. 11:399-404, 1984). The cells express the gene for *Androgen Receptor* (*AR*) at high levels.

PCR primers were used to amplify a segment of the *AR* gene to be used as the genetic probe. The 750bp *AR* PCR fragment was prepared by PCR amplification using primers: ARCS1 5'-TGAAGCAGGGATGACTCTGGG-3' (SEQ ID NO:5) and ARCAS4 5'-CTCGCAATAGGCTGCACGGAG-3' (SEQ ID NO:6) [position 2016 to 2766; Tilley et al, Proc. Natl. Acad. Sci. USA 86(1):327-331 (1989)]. The 750bp fragment generated with these primers was subcloned into the *Sma*I site of Bluescript plasmid vector (Stratagene, USA) and plasmid DNA prepared. The insert was isolated following restriction digestion with *Eco*RI and *Bam*HI and the isolated insert fragment was labelled with

digoxigenin-11-dUTP, using the random-primed labelling kit from Boehringer Mannheim (DIG-High Prime kit, 1995 Catalogue No. 1-585-606) and following the manufacturers instructions included in the kit.

LNCaP cell culture lines were grown and harvested using known methods (*In "Culture of Animal Cells: a manual of basic technique"* (1987), Freshney, R.I. (ed., Wiley-Lis, New York, USA).

Approximately 1000 LNCaP cells were added to a 20 ml sample of normal human male blood cells. Three other human male blood samples from patients with benign prostatic hyperplasia were used (approx. 10 mls each), but no LNCaP cells were added to these samples. The blood samples were then incubated with lysis buffer (0.1M NH_4Cl, 15mM $NaHCO_3$, 0.1mM Na_2EDTA) to lyse the red blood cells, centrifuged at 400g for 5 min, incubated a second time in lysis buffer and then washed with 50 ml cold saline. The white blood cells were centrifuged at 400g for 10 minutes in a clinical centrifuge. The cells were fixed by resuspending in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Centrifugation was repeated at 400g to pellet the cells. The excess fixative was withdrawn by aspiration. The cell pellet was washed in PBS and again centrifuged to pellet the cells. The PBS was removed by aspiration and the cells washed once more.

The cells were then permeabiulized by treatment with Proteinase K at 100 μg/ml for 5 min at 37°C. Permeabilization

was stopped with 0.2% (w/v) glycine in PBS for 2 min at room temperature and then the cells were washed twice in PBS as described above. The cells were then post-fixed in 4% paraformaldehyde as described above. The cells were washed two more times with PBS and pelleted as described above.

Cells were resuspended in hybridization buffer containing 50% formamide, 2xSSC, 25mM NaH_2PO_4 pH 7.4, 5% dextran sulphate, 250 g/ml sonicated, denatured salmon sperm DNA and the detection agent comprising a digoxigenin-labelled hybridization genetic probe at a concentration of 5 ng/μl. Prior to resuspending the cells, the labelled genetic probe, in hybridization buffer, was denatured by incubating the mixture at 80°C for 10 min followed by snap chilling on ice for 5 min. Hybridization was carried out for 16 hours at 37°C. Cells were pelleted at 400g in a clinical centrifuge and excess hybridization solution was removed. The cells were washed twice in 0.5xSSC twice at room temperature for 5 min and then washed a third time in 0.5xSSC at 37°C for 15 min. The cells were again pelleted and washed in PBS for 5 min.

Specific cells were then concentrated. A one in 500 dilution of a mouse anti-digoxigenen antibody (approx. 0.4 g) (1995 Cat No. 1-333-062, Boehringer Mannheim, Germany) containing 10% v/v sheep serum was added to the cells. The cells were incubated in the presence of the antibody for 3 hours at 4°C. The cells were then pelleted and excess antibody removed by aspiration and the cells washed twice in PBS as described above. The cell pellet was resuspended in 20μl of rat-anti-mouse IgG1-microbeads (1992 Cat No. 27-01,

Miltenyi Biotec GmbH, Germany) and incubated at 4°C for 15 min. Magnetic columns (1992 Cat No. 211-02, Type A2, Miltenyi Biotec GmbH, Germany) were pretreated by passing a mixture of ethanol/ethylsulfate/isopropyl alcohol/siloxane through the column and then rinsing the column with PBS. The column was then placed into a strong magnetic field (1992 Cat No. 231-02, MACS Separator, Miltenyi Biotec GmbH, Germany) and the magnetic bead:cell suspension was loaded onto the column then washed with 5 ml of PBS/0.01% sodium azide/1%BSA buffer. The column was removed from the magnetic field and backwashed with 2 ml of the PBS/0.01% sodium azide/1% BSA buffer to dislodge any cells that were non-specifically bound to the column. The column was placed back into the magnetic field and the cells allowed to migrate back onto the wire-mesh. The washing and backwashing step was repeated four times. Cells retained by the magnet after washing and backwashing were eluted by removing the column from the magnet and passing 10 ml of buffer through the column. This elution was repeated once more. The eluted cells were then centrifuged at 400g for 5 min to pellet the cells.

Pelleted cells were resuspended in approximately 50 μl of PBS buffer and then deposited onto a microscope slide and air dried. Cells on the slides were incubated in 20% normal sheep serum for 30 min at 20°C to block non-specific binding of the secondary antibody. Slides were briefly rinsed in PBS. A labelled secondary antibody, anti-digoxigenin-rhodamine Fab fragments from sheep (1995 Cat No. 1-207-750, Boehringer Mannheim, Germany), was applied to the cells at a dilution of 1:10 and incubated for 60 min at 20°C. The slides were washed

twice in PBS containing 0.1% v/v Nonidet P40 (Boehringer Mannheim, Germany). Anti-fade mountant (90% v/v glycerol 0.1% v/v p-phenylenediamine) was added to the cell samples and then a coverslip was applied. Cells were then detected with fluorescence microscopy using a 615nm emission wavelength filter and the total number of strongly fluorescent cells determined.

TABLE 3 **Recovery of *AR* positive cells in a model system and from patient samples.**

Sample	Number of LNCaP Cells Added[a]	Number of cells counted[b]
Normal male	1000	852
Patient 981	0	18
Patient 982	0	123
Patient 983	0	12

Notes:

[a] Number of LNCaP cells added.

[b] Number of strongly fluorescent cells counted following in situ hybridization with the 750bp *AR* probe and solid phase enrichment.

Table 3 shows that LNCaP cells were recovered from a mixed population of cells in normal human male blood using the 750bp *AR* digoxigenin-labelled genetic probe as a detecting agent and a superparamagnetic particle as the solid phase support for enrichment. Patient samples 981, 982 and 983 show that *AR* positive cells could be detected in peripheral blood from human males with prostatic disease.

All patents and publications in the specification are indicative of the level of ordinary skill in the art to which the invention pertains. All patents and publications herein are incorporated by reference to the same extent as if each individual patent and publication was specifically and individually indicated by reference.

It will be apparent to one of ordinary skill in the art that many changes and modifications can be made in the invention without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: KALIONIS, Bill

(ii) TITLE OF INVENTION: SOLID PHASE ENRICHMENT OF INTACT CELLS USING INTRACELLULAR CONSTITUENTS

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1 Little Collins Street
(C) CITY: Melbourne
(D) STATE: Victoria
(E) COUNTRY: Australia
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 17-Jan-1997

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/010113
(B) FILING DATE: 17-Jan-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: SLATTERY, John M.
(B) REGISTRATION NO:
(C) REFERENCE/DOCKET NUMBER: 1870760

(ix) TELECOMMUNICATION INFORMATION:
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2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

(G/C) AGGT (C/G) (A/C) A (A/G) C TGCAG (C/G) AGTC T 21

2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGAGACTGTGAGAGTGGTG 19

2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGTCTTCCGGGTGGAGC 17

2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTCACTATCAGCGCTGC 17

2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGAAGCAGGGATGACTCTGG G 21

2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCGCAATAG GCTGCACGGA G 21

CLAIMS:

1. An enriched cell complex, said enriched cell complex comprising:
   (a) a specific cell having a selected intracellular constituent;
   (b) a detecting agent hybridized to said selected intracellular constituent of said specific cell; and
   (c) a solid phase support coupled to said detecting agent.

2. The enriched cell complex according to claim 1 wherein said selected intracellular constituent is a nucleic acid.

3. The enriched cell complex according to claim 2 wherein said nucleic acid is amplified prior to contacting with said detecting agent.

4. The enriched cell complex according to claim 3 wherein said amplified nucleic acid further comprises a label which is detectable by said detecting agent.

5. The enriched cell complex according to claim 1 wherein said selected intracellular consituent is an amino acid, polypeptide or protein.

6. The enriched cell complex according to claim 1 wherein said detecting agent is an antibody.

7. The enriched cell complex according to claim 1 wherein said detecting agent is a genetic probe.

8. A method of solid phase enrichment of at least one specific cell in a mixed cell population, said method comprising steps of:

(a) contacting a mixed cell population with at least one detecting agent which binds to a selected intracellular constituent of said specific cell in said mixed cell population; and

(b) concentrating said specific cell having said selected intracellular constituent bound by said detecting agent utilizing a solid phase support system.

9. The method according to claim 8 wherein mixed cell population is fixed with a fixing agent and permeabilized with a permeabilizing agent, prior to contacting with said detecting agent.

10. The method according to claim 8 wherein said selected intracellular constituent is amplified prior to contacting with said detecting agent.

11. The method according to claim 8 wherein said selected intracellular constituent is in the cytoplasm of said specific cell.

12. The method according to claim 8 wherein said selected intracellular constituent is a nucleic acid.

13. The method according to claim 12 wherein said nucleic acid is amplified prior to contacting with said detecting agent.

14. The method according to claim 13 wherein said amplified nucleic acid further comprises a label which is detectable by said detecting agent.

15. The method according to claim 8 wherein said intracellular constituent is an amino acid, polypeptide or protein.

16. The method according to claim 8 wherein said detecting agent is an antibody.

17. The method according to claim 8 wherein said detecting agent is a genetic probe.

18. The method according to claim 8 further comprising the steps of:

(a) identifying said specific cell with an identifying agent; and

(b) visualizing said specific cell with a signal generating system.

19. A kit for enrichment of at least one specific cell from a mixed cell population based on detection of an intracellular constituent of said specific cell, said kit comprising:

(a) a detecting agent which detects said intracellular constituent of a said specific cell; and

(b) a solid phase support system.

20. The kit according to claim 19, further comprising:

(c) a fixing agent; and

(d) a permeabilizing agent.

21. The kit according to claim 19, further comprising:

(e) an identifying agent; and

(f) a signal generating system.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00020

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N-5/00 C12N-11/00, C12Q-1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IC6: C12N-011, C12Q-001/68-:keywords as below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Medline: see keywords below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Keywords: (intracellular, cytoplasmic) permeabilize, immobilize, hybridization WPAT, USPM, JAPIO, Chem Abs

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A1, 93/22053 (TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 11 November 1993, see particularly example 10	
A	Analytical Biochemistry volume 228, 1995, pages 252-258 Gibellini, D. *et al*	

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
	24 MAR 1997
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **JOHN ASHMAN** Telephone No.: (06) 283 2364

Form PCT/ISA/210 (second sheet) (July 1992) copyak

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00020

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00020

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00020

Box

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 97/00020

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9222053	AU	17625/92	BR	9205581	CA	2095919
		EP	586452	GB	9111621	GB	9211223
		GB	2256395	IL	102049	JP	6508935

END OF ANNEX